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(71) Applicant *(for all designated States except US)*: ADPROTECH PLC [GB/GB]; Unit 3, Number 2 Orchard Road, Royston, Herts. SG8 5HD (GB).

(72) Inventors; and
(75) Inventors/Applicants *(for US only)*: SMITH, Richard, Anthony, Godwin [GB/GB]; SmithKline Beecham Pharmaceuticals, New Frontiers Science Park South, Third Avenue, Harlow, Essex CM19 5AW (GB). DODD, Ian [GB/GB]; SmithKline Beecham Pharmaceuticals, New Frontiers Science Park South, Third Avenue, Harlow, Essex CM19 5AW (GB). MOSSAKOWSKA, Danuta, Ewa, Irena [GB/GB]; SmithKline Beecham Pharmaceuticals, New Frontiers Science Park South, Third Avenue, Harlow, Essex CM19 5AW (GB).

(74) Agent: DAVIES, Jonathan, Mark; Reddie & Grose, 16 Theobalds Road, London WC1X 8PL (GB).

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(57) Abstract

Soluble derivatives of soluble polypeptides incorporating membrane binding elements, their use in therapy and methods and intermediates including peptide membrane binding elements.

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CONJUGATES OF SOLUBLE PEPTIDIC COMPOUNDS WITH MEMBRANE-BINDING AGENTS

This invention relates to polypeptide derivatives, their use in therapy and methods and intermediates for their production.

Essentially all protein drugs are administered as solutions and function *in vivo* in the solution phase. In biochemistry and pharmacology, however, a large number of control and mediator proteins are associated with or function within or on the plasma membranes of cells. Except for soluble, truncated versions of one class of these molecules, no membrane-associated proteins have been developed as therapeutic agents. There are two main reasons for this situation. Firstly, overexpression of proteins that are retained in the membranes of the producer cells is limited by the the low capacity of membranes for proteins and often by the toxic effects of retention when expression is intrinsically efficient. Secondly, extraction of these proteins from membranes requires detergents or organic solvents, often results in inactivation of the protein, leads to difficulties in achieving the high purity needed for drug use and usually gives a product which is hard to formulate for intravenous administration. In addition, retention of very hydrophobic membrane anchoring elements may cause proteins to associate strongly with lipid-binding proteins in blood when administered intravenously thus preventing access to cell membranes.

Soluble, truncated versions of membrane-associated proteins overcome the production difficulties associated with full length proteins. However such truncated molecules lack the membrane binding capability and specificity of the full length proteins which properties may be advantageous or even essential to the desired therapeutic activity.

The main classes of interaction of proteins with membranes can be summarised as follows:

1. Direct and specific interactions with phospholipid head groups or with other hydrophilic regions of complex lipids or indirectly with proteins already inserted in the membrane. The latter may include all the types of intrinsic membrane protein noted below and such interactions are usually with extracellular domains or sequence loops of the membrane proteins;

2. Through anchoring by a single hydrophobic transmembrane helical region near the terminus of the protein. These regions commonly present a hydrophobic face around the entire circumference of the helix cylinder and transfer of this structure to the hydrophilic environment of bulk water is energetically unfavourable.

3. Further anchoring is often provided by a short sequence of generally cationic aminoacids at the cytoplasmic side of the membrane, C-terminal to the transmembrane helix;

4. Through the use of multiple (normally 2-12 and commonly 4,7 and 10) transmembrane regions which are usually predicted to be helical or near-helical. Although these regions are normally hydrophobic overall, they frequently show some amphipathic behaviour - an outer hydrophobic face and an inner more hydrophilic one being identifiable within a helix bundle located in the lipid bilayer;

5. Through postranslationally linked phosphatidyl inositol moeities (GPI-anchors). These are generated by a specific biosynthetic pathway which recognises and removes a specific stretch of C-terminal aminoacids and creates a membrane-associating diacyl glycerol unit linked *via* a hydrophilic carbohydrate spacer to the polypeptide;

6. In a related process, single fatty acid groups such as myristoyl, palmitoyl or prenyl may be attached postranslationally to one or more sites in a protein (usually at N- or C-termini). Again, amino acids (such as the C-terminal CAAX box in *Ras* proteins) may be removed.

Artificial membranes are considered to be lipid complexes that mimic the basic properties of the cell membrane, i.e., a lipid vacuole with an aqueous interior and a surface chemistry that resembles the cell membrane. The artificial membrane typically contains phospholipids or mimics thereof and may be unilemellar or bilemellar and the outer surface will contain charged groups similar to the choline groups of the most abundant phospholipid. The prototype artificial membrane is known as a liposome and the technologies for the construction of liposomes including the incorporation of therapeutically useful agents into them is well known to those in the art. Liposomes have been evaluated in a number of disease states and liposomes containing the anti-fungal Amphotericin are commercially available. In addition, proteoliposomes have been described. For example, the use of immunoliposomes encapsulating amphotericin B has been reported to be of benefit in the treatment of experimental fungal infections in animal models (e.g. Hospenthal, D. et al (1989) J. Med. Microbiol. **30** 193-197; Dromer, F. et al (1990) Antimicrob. Agents Chemother. **34** 2055-2060).

Mimics of natural or artificial membranes are often related in structure and will mimic one or more properties of the membrane. One such example is the provision of an artificial surface having pendant groups which mimic the phospholipid zwitterionic groups which are found on the outside of cell surfaces. For example WO92/06719 (Biocompatibles Limited) discloses natural and synthetic phospholipids which may be coated on an artificial surface, e.g. a device which, in use, will come into contact with protein-containing or biological fluids, to provide improved biocompatibility and haemocompatibility and WO 94/16749 discloses additional zwitterionic groups that may be used to improve biocompatibility in a similar way.

The present invention provides a soluble derivative of a soluble polypeptide, said derivative comprising two or more heterologous membrane binding elements with low membrane affinity covalently associated with the polypeptide which elements are capable of interacting, independently and with thermodynamic additivity, with components of cellular or artificial membranes exposed to extracellular fluids.

By 'heterologous' is meant that the elements are not found in the native full length protein from which a soluble protein may be derived.

By 'soluble polypeptide' is meant a truncated derivative of a full length protein which lacks its natural membrane binding capability, and/or a polypeptide which has a solubility level in aqueous media of > 100μg/ml.

By 'membrane binding element with low membrane affinity' is meant that the element has only moderate affinity for membranes, that is a dissociation constant greater than 0.1μM, preferably 1μM-1mM. The elements preferably have a size <5kDa.

The derivative should incorporate sufficient elements with low affinities for membrane components to result in a derivative with a high (preferably 0.01 - 10nM dissociation constant) affinity for specific membranes. The elements combine so as to create an overall high affinity for the particular target membrane but the combination lacks such high affinity for other proteins for which single elements may be (low-affinity) ligands.

The elements should be chosen so as to retain useful solubility in pharmaceutial formulation media, preferably >100μg/ml. Preferably at least one element is hydrophilic.

The invention thus promotes localisation of a therapeutic protein at cellular membranes and thereby provides one or more of several biologically significant effects with potential therapeutic advantages including:

Potency: If the protein is a receptor and an agonist or antagonist activity is localised on the same surface as the receptor itself, an increase in effective concentration may result from the reduction in the diffusional degrees of freedom.

Pharmacokinetics and dosing frequency: Interaction of a derivatised protein with long-lived cell types or serum proteins would be expected to prolong the plasma residence time of the protein and produce a depot effect through deposition on cell surfaces.

Specificity: Many clinically important pathological processes are associated with specific cell types and tissues (for example the vascular endothelium and the recruitment thereto of neutrophils bearing the sialyl Lewis ^{x} antigen to ELAM-1, see below). Hence targeting the modified protein to regions of membrane containing

pathology-associated membrane markers may improve the therapeutic ratio of the protein targeted.

The derivatives of the invention may be used in association with artificial membranes or mimics thereof to allow delivery of the therapeutic protein to sites where it will provide therapeutic benefit. For example, polypeptides associated with liposomes formed by contacting liposomes with a derivative of the invention may be more stable than the free polypeptide. The liposome may incorporate a therapeutic agent, for example an antiflammatory or cytotoxic agent. The polypeptide derivative of the invention may thus be used to target the therapeutic agent. When the polypeptide is itself a therapeutic agent, the liposome incorporated therapeutic agent may be used to enhance further the efficacy or tolerability of the therapy.

Association of derivatives of the invention with mimics of cell membranes may be used to concentrate the therapeutic protein at sites where therapeutically useful concentrations of underivatised protein might be difficult to achieve. For example, indwelling medical devices coated with mimics of the phospholipid zwitterionic groups which are found on the outside of cell surfaces, such as those disclosed in WO92/06719 and WO 94/16749, may be additionally treated with derivatives of the invention. For example complement inhibitors derivatised in accordance with the invention could be incorporated into the outer surface of indwelling catheters or hip replacements or heart valves in order to minimise development of inflammation associated with these operations.

It will be appreciated that all associations of heterologous amino acid sequences with a polypeptide which is a soluble derivative of a human protein will need to be assessed for potential immunogenicity, particularly where the amino acid sequence is not derived from a human protein. The problem can be minimised by using sequences as close as possible to known human ones and through computation of secondary structure and antigenicity indices.

Examples of protein therapeutic agents which may be modified according to the invention include but are not restricted to the following:

Base Protein	Cell Target	Therapeutic Application
IL-4 Y124D mutein	B-cells	Anti-allergy (IL-4 antagonist)
Plasminogen activators e.g. Prourokinase, streptokinase, tissue-type plasminogen activator, reteplase	Erythrocytes, vascular endothelium	Prevention of venous thrombosis
Leptin	Choroid plexus, Hypothalamus	Weight loss (agonist)
Complement inhibitors*	Vascular endothelium, Myocytes, Erythrocytes, Lymphocytes	Ischaemic injury, transplantation, inflammation
scFv antibody against cytokines (IL-1, IL-, IL-5, IL-6)	Eosinophils	Asthma, allergic disease
Protein C	Vascular endothelium	Prevention of venous thrombosis
Antibodies against CD4, B7/CD28, CD3/TCR, CD11b(CR3)	Lymphocytes	Immunosuppression
Interferon-β and derivatives	Lymphocytes	Immunomodulation, multiple sclerosis

*Complement regulatory proteins e.g.: CR1 (CD35); DAF (CD55); MCP (CD46); CD59; Factor H; and C4 binding protein; and hybrids or muteins thereof such as CR1-CD59 (S.G.El Feki and D.T.Fearon Molecular Immunology **33** (supp 1). p 57, 1996), MCP-DAF (P.J.Higgins *et al*, J.Immunology. **158**, 2872-2881,1997) and soluble CR1 polypeptide fragments.

The derivative preferably comprises two to eight, more preferably two to four membrane binding elements.

Membrane binding elements are preferably selected from: fatty acid derivatives such as fatty acyl groups; basic amino acid sequences; ligands of known integral membrane proteins; sequences derived from the complementarity-determining region of monoclonal antibodies raised against epitopes of membrane proteins; membrane binding sequences identified through screening of random chemical or peptide libraries.

The selection of suitable combination of membrane binding elements will be guided by the nature of the target cell membrane or components thereof.

Suitable fatty acid derivatives include myristoyl (12 methylene units) which is insufficiently large or hydrophobic to permit high affinity binding to membranes. Studies with myristoylated peptides (eg R.M.Peitzsch & S.McLaughlin, Biochemistry, 32, 10436-10443, 1993)) have shown that they have effective dissociation constants with model lipid systems of ~10^{-4} M and around 10 of the 12 methylene groups are buried in the lipid bilayer. Thus, aliphatic acyl groups with about 8 to 18 methylene units, preferably 10-14, are suitable membrane binding elements. Other examples of suitable fatty acid derivatives include long-chain (8-18, preferably 10-14 methylene) aliphatic amines and thiols, steroid and farnesyl derivatives.

Membrane binding has been found to be associated with limited (single-site) modification with fatty acyl groups when combined with a cluster of basic aminoacids in the protein sequence which may interact with acidic phospholipid head groups and provide the additional energy to target membrane binding. This combination of effects has been termed the 'myristoyl-electrostatic switch' (S.McLaughlin and A.Aderem, TIBS, 20,272-276, 1994; J.F.Hancock *et al*, Cell, 63, 133-139,1990). Thus, a further example of suitable membrane binding elements are basic aminoacid sequences such as those found in proteins such as *Ras* and MARCKS (myristoylated alanine-rich C-kinase substrate, P.J. Blackshear, J. Biol. Chem., 268, 1501-1504, 1993) which mediate the electrostatic 'switch' through reversible phosphorylation of serine residues within the sequence and a concomitant neutralisation of the net positive charge. Such sequences include but are not restricted to consecutive sequences of Lysine and Arginine such as (Lys)n where n is from 3 to 10, preferably 4 to 7.

Suitable examples of amino acid sequences comprising basic amino acids include:

i) DGPKKKKKKSPSKSSG

ii) GSSKSPSKKKKKKPGD

iii) SPSNETPKKKKKRFSFKKSG

iv) DGPKKKKKKSPSKSSK

v) SKDGKKKKKKSKTK

(N-terminus on left)

Sequences i) to v) are examples of electrostatic switch sequences.

Examples of amino acid sequences derived from ligands of known integral membrane proteins include RGD-containing peptides such as GRGDSP which are ligands for the $\alpha_{IIb}\beta_3$ integrin of human platelet membranes. Another example is DGPSEILRGDFSS derived from human fibrinogen alpha chain, which binds to the GpIIb/IIIa membrane protein in platelets.

Further examples of such sequences include those known to be involved in interactions between membrane proteins such as receptors and the major

histocompatibility complex. An example of such a membrane protein ligand is the sequence GNEQSFRVDLRTLLRYA which has been shown to bind to the major histocompatibility complex class 1 protein (MHC-1) with moderate affinity (L.Olsson *et al*, Proc. Natl .Acad.Sci.USA. 91, 9086-909, 1994).

Yet further examples of such sequences employ a membrane insertive address specific for T-cells. Such sequence is derived from the known interaction of the transmembrane helix of the T-cell antigen receptor with CD3 (Nature Medicine 3, 84-88,1997). Examples are peptides containing the sequence GFRILLLKV such as:
SAAPSSGFRILLLKV
AAPSVIGFRILLLKVAG

An example of a ligand for an integral membrane protein is the carbohydrate ligand Sialyl Lewis^x which has been identified as a ligand for the integral membrane protein ELAM-1 (M.L.Phillips *et al*, Science, 250, 1130-1132, 1990 & G.Walz *et al*, *Ibid*, 250, 1132-1135,1990).

Sequences derived from the complementarity-determining regions of monoclonal antibodies raised against epitopes within membrane proteins (see, for example, J.W.Smith *et al*, J.Biol.Chem. 270, 30486-30490, 1995) are also suitable membrane binding elements, as are binding sequences from random chemical libraries such as those generated in a phage display format and selected by biopanning operations *in vitro* (G.F.Smith and J.K.Scott, Methods in Enzymology, 217H, 228-257,1993) or *in vivo* (R.Pasqualini & E.Ruoslahti, Nature, 380, 364-366, 1996).

Optionally, conditional dissociation from the membrane may be incorporated into derivatives of the invention using mechanisms such as pH sensitivity (electrostatic switches), regulation through metal ion binding (using endogenous Ca^{2+}, Zn^{2+} and incorporation of ion binding sites in membrane binding elements) and protease cleavage (e.g plasminolysis of lysine-rich membrane binding sequences to release and activate prourokinase)

Preferred derivatives of this invention have the following structure:

$[P]-\{L-[W]\}_n-X$

in which:
P is the soluble polypeptide,
each **L** is independently a flexible linker group,
each **W** is independently a peptidic membrane binding element,
n is an integer of 1 or more and
X is a peptidic or non-peptidic membrane-binding entity which may be covalently linked to any **W**.

Peptidic membrane binding elements are preferably 8 to 20 amino acids long and elements W are preferably located sequentially either at the N or C terminus of the soluble polypeptide. The amino acid sequences are linked to one another and to the soluble peptide by linker groups which are preferably selected from hydrophilic and/or flexible aminoacid sequences of 4 to 20 aminoacids; linear hydrophilic synthetic polymers; and chemical bridging groups.

Peptide linkages may be made chemically or biosynthetically by expression of appropriate coding DNA sequences. Non peptide linkages may be made chemically or enzymatically by post-translational modification.

The polypeptide portion of the derivatives of the invention may be prepared by expression in suitable hosts of modified genes encoding the soluble polypeptide of interest plus one or more peptidic membrane binding elements and optional residues such as cysteine to introduce linking groups to facilitate post translational derivatisation with additional membrane binding elements.

In a further aspect, therefore, the invention provides a process for preparing a derivative according to the invention which process comprises expressing DNA encoding the polypeptide portion of said derivative in a recombinant host cell and recovering the product and thereafter post translationally modifying the polypeptide to chemically introduce membrane binding elements.

In particular, the recombinant aspect of the process may comprise the steps of:

i) preparing a replicable expression vector capable, in a host cell, of expressing a DNA polymer comprising a nucleotide sequence that encodes said polypeptide portion;

ii) transforming a host cell with said vector;

iii) culturing said transformed host cell under conditions permitting expression of said DNA polymer to produce said polypeptide; and

iv) recovering said polypeptide.

Where the polypeptide portion is novel, the DNA polymer comprising a nucleotide sequence that encodes the polypeptide portion as well as the polypeptide portion itself and S-derivatives thereof, also form part of the invention. In particular the invention provides a polypeptide portion of a derivative of the invention comprising the soluble peptide linked by a peptide bond to one peptidic membrane binding element and/or including a C-terminal cysteine, and DNA polymers encoding the polypeptide portion.

The recombinant process of the invention may be performed by conventional recombinant techniques such as described in Sambrook *et al.*, Molecular Cloning : A

laboratory manual 2nd Edition. Cold Spring Harbor Laboratory Press (1989) and DNA Cloning vols I, II and III (D. M. Glover ed., IRL Press Ltd).

The invention also provides a process for preparing the DNA polymer by the condensation of appropriate mono-, di- or oligomeric nucleotide units.

The preparation may be carried out chemically, enzymatically, or by a combination of the two methods, *in vitro* or *in vivo* as appropriate. Thus, the DNA polymer may be prepared by the enzymatic ligation of appropriate DNA fragments, by conventional methods such as those described by D. M. Roberts *et al.*, in Biochemistry 1985, 24, 5090-5098.

The DNA fragments may be obtained by digestion of DNA containing the required sequences of nucleotides with appropriate restriction enzymes, by chemical synthesis, by enzymatic polymerisation, or by a combination of these methods.

Digestion with restriction enzymes may be performed in an appropriate buffer at a temperature of 20°-70°C, generally in a volume of 50µl or less with 0.1-10µg DNA.

Enzymatic polymerisation of DNA may be carried out *in vitro* using a DNA polymerase such as DNA polymerase 1 (Klenow fragment) in an appropriate buffer containing the nucleoside triphosphates dATP, dCTP, dGTP and dTTP as required at a temperature of 10°-37°C, generally in a volume of 50µl or less.

Enzymatic ligation of DNA fragments may be carried out using a DNA ligase such as T4 DNA ligase in an appropriate buffer at a temperature of 4°C to 37°C, generally in a volume of 50µl or less.

The chemical synthesis of the DNA polymer or fragments may be carried out by conventional phosphotriester, phosphite or phosphoramidite chemistry, using solid phase techniques such as those described in 'Chemical and Enzymatic Synthesis of Gene Fragments - A Laboratory Manual' (ed. H.G. Gassen and A. Lang), Verlag Chemie, Weinheim (1982), or in other scientific publications, for example M.J.Gait, H.W.D. Matthes M. Singh, B.S. Sproat and R.C. Titmas, Nucleic Acids Research, 1982, 10, 6243; B.S. Sproat and W. Bannwarth, Tetrahedron Letters, 1983, 24, 5771; M.D. Matteucci and M.H. Caruthers, Tetrahedron Letters, 1980, 21, 719; M.D. Matteucci and M.H. Caruthers, Journal of the American Chemical Society, 1981, 103, 3185; S.P. Adams *et al.*, Journal of the American Chemical Society, 1983, 105, 661; N.D. Sinha, J. Biernat, J. McMannus and H. Koester, Nucleic Acids Research, 1984, 12, 4539; and H.W.D. Matthes *et al.*, EMBO Journal, 1984, 3, 801. Preferably an automated DNA synthesiser (for example, Applied Biosystems 381A Synthesiser) is employed.

The DNA polymer is preferably prepared by ligating two or more DNA molecules which together comprise a DNA sequence encoding the polypeptide.

The DNA molecules may be obtained by the digestion with suitable restriction enzymes of vectors carrying the required coding sequences.

The precise structure of the DNA molecules and the way in which they are obtained depends upon the structure of the desired product. The design of a suitable strategy for the construction of the DNA molecule coding for the polypeptide is a routine matter for the skilled worker in the art.

In particular, consideration may be given to the codon usage of the particular host cell. The codons may be optimised for high level expression in *E. coli* using the principles set out in Devereux *et al.*, (1984) Nucl. Acid Res., 12, 387.

The expression of the DNA polymer encoding the polypeptide in a recombinant host cell may be carried out by means of a replicable expression vector capable, in the host cell, of expressing the DNA polymer. Novel expression vectors also form part of the invention.

The replicable expression vector may be prepared in accordance with the invention, by cleaving a vector compatible with the host cell to provide a linear DNA segment having an intact replicon, and combining said linear segment with one or more DNA molecules which, together with said linear segment, encode the polypeptide, under ligating conditions.

The ligation of the linear segment and more than one DNA molecule may be carried out simultaneously or sequentially as desired.

Thus, the DNA polymer may be preformed or formed during the construction of the vector, as desired. The choice of vector will be determined in part by the host cell, which may be prokaryotic, such as *E. coli*, or eukaryotic, such as mouse C127, mouse myeloma, chinese hamster ovary, fungi e.g. filamentous fungi or unicellular 'yeast' or an insect cell such as Drosophila. The host cell may also be in a transgenic animal. Suitable vectors include plasmids, bacteriophages, cosmids and recombinant viruses derived from, for example, baculoviruses or vaccinia.

The DNA polymer may be assembled into vectors designed for isolation of stable transformed mammalian cell lines expressing the fragment e.g. bovine papillomavirus vectors in mouse C127 cells, or amplified vectors in chinese hamster ovary cells (DNA Cloning Vol. II D.M. Glover ed. IRL Press 1985; Kaufman, R.J. *et al.*. Molecular and Cellular Biology 5, 1750-1759, 1985; Pavlakis G.N. and Hamer, D.H. Proceedings of the National Academy of Sciences (USA) 80, 397-401, 1983; Goeddel, D.V. *et al.*,European Patent Application No. 0093619, 1983).

The preparation of the replicable expression vector may be carried out conventionally with appropriate enzymes for restriction, polymerisation and ligation of the DNA, by procedures described in, for example, Sambrook *et al.*,cited above.

Polymerisation and ligation may be performed as described above for the preparation of the DNA polymer. Digestion with restriction enzymes may be performed in an appropriate buffer at a temperature of 20°-70°C, generally in a volume of 50µl or less with 0.1-10µg DNA.

The recombinant host cell is prepared, in accordance with the invention, by transforming a host cell with a replicable expression vector of the invention under transforming conditions. Suitable transforming conditions are conventional and are described in, for example, Sambrook *et al.*, cited above, or "DNA Cloning" Vol. II, D.M. Glover ed., IRL Press Ltd, 1985.

The choice of transforming conditions is determined by the host cell. Thus, a bacterial host such as *E.coli*, may be treated with a solution of $CaCl_2$ (Cohen *et al.*,Proc. Nat. Acad. Sci., 1973, 69, 2110) or with a solution comprising a mixture of RbCl, $MnCl_2$, potassium acetate and glycerol, and then with 3-[N-morpholino]-propane-sulphonic acid, RbCl and glycerol or by electroporation as for example described by Bio-Rad Laboratories, Richmond, California, USA, manufacturers of an electroporator. Mammalian cells in culture may be transformed by calcium co-precipitation of the vector DNA onto the cells or by using cationic liposomes.

The invention also extends to a host cell transformed with a replicable expression vector of the invention.

Culturing the transformed host cell under conditions permitting expression of the DNA polymer is carried out conventionally, as described in, for example, Sambrook *et al.*, and "DNA Cloning" cited above. Thus, preferably the cell is supplied with nutrient and cultured at a temperature below 45°C.

The protein product is recovered by conventional methods according to the host cell. Thus, where the host cell is bacterial such as *E. coli* and the protein is expressed intracellularly, it may be lysed physically, chemically or enzymatically and the protein product isolated from the resulting lysate. Where the host cell is mammalian, the product is usually isolated from the nutrient medium.

Where the host cell is bacterial, such as *E. coli*, the product obtained from the culture may require folding for optimum functional activity. This is most likely if the protein is expressed as inclusion bodies. There are a number of aspects of the isolation and folding process that are regarded as important. In particular, the polypeptide is preferably partially purified before folding, in order to minimise formation of aggregates with contaminating proteins and minimise misfolding of the polypeptide. Thus, the removal of contaminating *E. coli* proteins by specifically isolating the inclusion bodies and the subsequent additional purification prior to folding are important aspects of the procedure.

The folding process is carried out in such a way as to minimise aggregation of intermediate-folded states of the polypeptide. Thus, careful consideration needs to be given to, among others, the salt type and concentration, temperature, protein concentration, redox buffer concentrations and duration of folding. The exact condition for any given polypeptide generally cannot be predicted and must be determined by experiment.

There are numerous methods available for the folding of proteins from inclusion bodies and these are known to the skilled worker in this field. The methods generally involve breaking all the disulphide bonds in the inclusion body, for example with 50mM 2-mercaptoethanol, in the presence of a high concentration of denaturant such as 8M urea or 6M guanidine hydrochloride. The next step is to remove these agents to allow folding of the proteins to occur. Formation of the disulphide bridges requires an oxidising environment and this may be provided in a number of ways, for example by air, or by incorporating a suitable redox system, for example a mixture of reduced and oxidised glutathione.

Preferably, the inclusion body is solubilised using 8M urea, in the presence of mercaptoethanol, and protein is folded, after initial removal of contaminating proteins, by addition of cold buffer. Suitable buffers may be identified using the techniques described in I.Dodd *et al*, 'Perspectives in Protein Engineering and Complementary Technologies' , Mayflower Publications, 66-69, 1995. A suitable buffer for many of the SCR constructs described herein is 20mM ethanolamine containing 1mM reduced glutathione and 0.5mM oxidised glutathione. The folding is preferably carried out at a temperature in the range 1 to 5^{o}C over a period of 1 to 4 days.

If any precipitation or aggregation is observed, the aggregated protein can be removed in a number of ways, for example by centrifugation or by treatment with precipitants such as ammonium sulphate. Where either of these procedures are adopted, monomeric polypeptide is the major soluble product.

If the bacterial cell secretes the protein, folding is not usually necessary.

The polypeptide portion of the derivative of the invention may include a C-terminal cysteine to facilitate post translational modification. A soluble polypeptide including a C-terminal cysteine also forms part of the invention. Expression in a bacterial system is preferred for proteins of moderate size (up to ~70kDa) and with <~8 disulphide bridges. More complex proteins for which a free terminal Cys could cause refolding or stability problems may require stable expression in mammalian cell lines (especially CHO). This will also be needed if a carbohydrate membrane binding element is to be introduced post-translationally. The use of insect cells infected with recombinant baculovirus encoding the polypeptide portion is also a useful general method for

preparing more complex proteins and will be preferred when it is desired to carry out certain post-translational processes (such as palmitoylation) biosynthetically (see for example, M.J.Page *et al* J.Biol.Chem. 264, 19147-19154, 1989)

A preferred method of handling proteins C-terminally derivatised with cysteine is as a mixed disulphide with mercaptoethanol or glutathione or as the 2-nitro, 5-carboxyphenyl thio- derivative as generally described below in Methods.

Peptide membrane binding elements may be prepared using standard solid state synthesis such as the Merrifield method and this method can be adapted to incorporate required non-peptide membrane binding elements such as N-acyl groups derived from myristic or palmitic acids at the N terminus of the peptide. In addition activation of an amino acid residue for subsequent linkage to a protein can be achieved during chemical synthesis of such membrane binding elements. Examples of such activations include formation of the mixed 2-pyridyl disulphide with a cysteine thiol or incorporation of an N-haloacetyl group. Both of these groups are capable of reaction with free thiols, through disulphide interchange and alkylation, respectively. Peptides can optionally be prepared as the C-terminal amide and/or with a conventional N-terminal blocking group such as acetyl.

The invention also provides a peptidic membrane binding element comprising one or more derivatisations selected from:

a terminal cysteine residue optionally activated at the thiol group;

an N-haloacetyl group (where halo signifies chlorine, bromine or iodine) located at the N-terminus of the the peptide or at an ε-amino group of a lysine residue;

an amide group at the C-terminus;

an N-terminal blocking group; and

a fatty acid N-acyl group at the N-terminus or at an ε-amino group of a lysine residue.

Chemical bridging groups and reagents suitable for their formation include those described in EP0109653, EP0152736, EP0155388 and EP0284413, incorporated herein by reference. The bridging group is generally of the formula:

$$-A-R-B- \qquad (I)$$

in which each of A and B, which may be the same or different, represents -CO-, $-C(=NH_2^+)-$, maleimido, -S- or a bond and R is a bond or a linking group containing one or more $-(CH_2)-$ or meta-, ortho- or para- disubstituted phenyl units, preferably ortho or para, optionally together with a hydrophilic portion.

Where the polypeptide portion of the derivative of the invention and a peptidic membrane binding element both include a C-terminal cysteine the chemical bridging group will take the form -S-S-. The bridge is generated by conventional disulphide

exchange chemistry, by activating a thiol on one polypeptide and reacting the activated thiol with a free thiol on the other polypeptide. Such activation procedures make use of disulphides which form stable thiolate anions upon cleavage of the S-S linkage and include reagents such as 2,2' dithiopyridine and 5,5'-dithio(2-nitrobenzoic acid, DTNB) which form intermediate mixed disulphides capable of further reaction with thiols to give stable disulphide linkages.

R may include moieties which interact with water to maintain the water solubility of the linkage and suitable moieties include -CO-NH-, -CO-NMe-, -S-S-, -CH(OH)-, $-SO_2-$, $-CO_2-$, $-(CH_2CH_2-O)_m-$ and -CH(COOH)- where m is an integer of 2 or more, or linear hydrophilic polymers such as polyethylene glycol, polypropylene glycol, polyglycine, polyalanine or polysarcosine.

Other examples of R include $-(CH_2)_r-$, $-(CH_2)_p-S-S-(CH_2)_q-$ and $-(CH_2)_p-CH(OH)-CH(OH)-(CH_2)_q-$, in which r is an integer of at least 2, preferably at least 4 and p and q are independently integers of at least 2

In a further aspect R may take the form -U-V-W- where U is $(CH_2)_2CONH(CH_2)_n$ in which n is an integer of 3 to 8, V is O, S, NR_a or NR_a-NR_a where each R_a is H or C_{1-6} alkyl, NH-O or O-NH, and W is benzyl substituted at the 2- or 4- position by the group B. In a preferred embodiment R is $(CH_2)_2CONH(CH_2)_nNH$-(4-phenyl) where n is an integer of 3 to 8.The bridging group of formula (I) may be derived from a linking agent of formula (II):

$$X-R_1-Y \qquad (II)$$

in which R_1 is a bond or a linking group and X and Y are functional groups reactable with surface amino acid groups, preferably a lysine or cysteine group, the N-terminal amino group, a catalytic serine hydroxyl or a protein attachment group, and X, R_1- and Y are chosen so as to generate the required bridging group -A-R-B-.

Preferred agents are those where X and Y are different, known as heterobifunctional agents. Each end of the agent molecule is reacted in turn with each polypeptide to be linked in separate reactions. Examples of heterobifunctional agents of formula (II) include:

N-succinimidyl 3-(2-pyridyldithio) propionate

succinimidyl 4-(N-maleimido) caproate

3-(2-pyridyl) methyl propionimidate hydrochloride

4'-amidinophenyl 4-N-[2-N-(3-[2-pyridyldithio]ethylcarbonyl)aminoethyl] aminobenzoate hydrochloride.

Other suitable agents are disclosed in EP0109653, EP0152736, EP0155388 and EP0284413, in particular those of formula (II) in EP0155388 and (III) in EP0284413 incorporated herein by reference.

In each case Y is capable of reacting with a thiol group on a polypeptide, which may be a native thiol or one introduced as a protein attachment group.

The protein attachment group is a functionality derived by modification of a polypeptide or protein with a reagent specific for one or more amino acid side chains, and which contains a group capable of reacting with a cleavable section on the other polypeptide. An example of a protein attachment group is a thiol group. An example of a cleavable section is a disulphide bond. Alternatively the cleavable section may comprise an α, β dihydroxy function.

As an example, the introduction of a free thiol function by reaction of a polypeptide with 2-iminothiolane, N-succinimidyl 3-(2-pyridyldithio) propionate (with subsequent reduction) or N-acetyl homocysteine thiolactone will permit coupling of the protein attachment group with a thiol-reactive Y structure. Alternatively, the protein attachment group can contain a thiol-reactive entity such as the 6-maleimidohexyl group or a 2-pyridyl-dithio group which can react with a free thiol in X. Preferably, the protein attachment group is derived from protein modifying agents such as 2-iminothiolane that react with lysine ε-amino groups in proteins.

When X represents a group capable of reacting directly with the amino acid side chain of a protein, it is preferably an N-succinimidyl group. When X represents a group capable of reacting with a protein attachment group, it is preferably a pyridylthio group. When X represents a group capable of reacting with a catalytic serine hydroxyl it is preferably an 4-amidinophenyl ester group optionally substituted by one or more electron withdrawing groups which increases the reactivity of the ester, of the kind contained in the compounds of formula (II) in EP0155388 and (III) in EP0284413.

In the above processes, modification of a polypeptide to introduce a protein attachment group is preferably carried out in aqueous buffered media at a pH between 3.0 and 9.0 depending on the reagent used. For a preferred reagent, 2-iminothiolane, the pH is preferably 6.5-8.5. The concentration of polypeptide is preferably high (> 10mg/ml) and the modifying reagent is used in a moderate (1.1- to 5-fold) molar excess, depending on the reactivity of the reagent. The temperature and duration of reaction are preferably in the range 0^{o}-40^{o}C and 10 minutes to 7 days. The extent of modification of the polypeptide may be determined by assaying for attachment groups introduced.

Such assays may be standard protein chemical techniques such as titration with 5,5'-dithiobis-(2-nitrobenzoic acid). Preferably, 0.5-3.0 moles of protein attachment group will be introduced on average per mole of polypeptide. The modified polypeptide may be separated from excess modifying agents by standard techniques such as dialysis, ultrafiltration, gel filtration and solvent or salt precipitation. The intermediate material may be stored in frozen solution or lyophilised.

Where the linking agent of formula (II) contains an amidino phenyl ester group X the agent is preferably first reacted with a polypeptide enzyme via the group X and the reaction is preferably carried out under the conditions described for the introduction of blocking groups in European Published Patent Application No. 0,009,879. Having been freed of excess reagent by suitable techniques such as high performance size exclusion chromatography or diafiltration, the acylated enzyme may then be reacted with the other polypeptide at a ratio of approximately 1:1 in a non-nucleophilic buffer at pH7.0-8.0 and 0°-30°C for up to 6h. However, it is preferable to conduct the coupling below 10°C (preferably 0°-4°C) in order to minimise the hydrolysis of the acylated enzyme.

Where a protein attachment group is introduced in this way, the bridging group (I) will be formed from a reaction of the linking agent (II) and the protein attachment group.

The polypeptides to be linked are reacted separately with the linking agent or the reagent for introducing a protein attachment group by typically adding an excess of the reagent to the polypeptide, usually in a neutral or moderately alkaline buffer, and after reaction removing low molecular weight materials by gel filtration or dialysis. The precise conditions of pH, temperature, buffer and reaction time will depend on the nature of the reagent used and the polypeptide to be modified. The polypeptide linkage reaction is preferably carried out by mixing the modified polypeptides in neutral buffer in an equimolar ratio. Other reaction conditions e.g. time and temperature, should be chosen to obtain the desired degree of linkage. If thiol exchange reactions are involved, the reaction should preferably be carried out under an atmosphere of nitrogen. Preferably, UV-active products are produced (eg from the release of pyridine 2-thione from 2-pyridyl dithio derivatives) so that coupling can be monitored.

After the linkage reaction, the polypeptide conjugate can be isolated by a number of chromatographic procedures such as gel filtration, ion-exchange chromatography, affinity chromatography or hydrophobic interaction chromatography. These procedures my be either low pressure or high performance variants.

The conjugate may be characterised by a number of techniques including low pressure or high performance gel filtration, SDS polyacrylamide gel electrophoresis or isoelectric focussing.

Membrane binding elements which are fatty acid derivatives are attached post translationally to a peptidic membrane binding element, preferably at the terminus of the polypetide chain. Preferably, where the recombinant polypeptide portion of the derivative of the invention contains the peptidic membrane binding element, it has a unique cysteine for coupling to the fatty acid derivative. Where the recombinant polypeptide has a cysteine residue, a thiol-derivative of the fatty acid is added to the

refolded recombinant protein at a late stage in purification (but not necessarily the final stage) and at a reagent concentration preferably below the critical micelle concentration. One of the fatty acid derivative and the recombinant peptide will have the thiol group activated as described above for thiol interchange reactions. The fatty acid derivative is preferably a C_{10-20} fatty acyl derivative of an aminoC_{2-6}alkane thiol (optionally C-substituted) such as N-(2-myristoyl) aminoethanethiol or N-myristoyl L-cysteine and forms part of the invention.

Suitable examples of hydrophilic synthetic polymers include polyethyleneglycol (PEG), preferably α,ω functionalised derivatives, more preferably α-amino , ω-carboxy-PEG of molecular weight between 400 and 5000 daltons which are linked to the polypeptide for example by solid-phase synthesis methods (amino group derivatisation) or by thiol-interchange chemistry.

Membrane binding elements derived from ligands of known integral membrane proteins, either amino acid sequences or carbohydrates, may be generated by post-translational modification using the glycosylation pathways of eukaryotic cells targeted to N-linked glycosylation sites in the peptide sequence.

Convenient generic final stage purification strategies are hydrophobic interaction chromatography (HIC) on C2-C8 media and cation exchange chromatography for separation of derivatised and underivatised proteins into which a hydrophobic-electrostatic switch combination has been inserted.

The derivatives of this invention are preferably administered as pharmaceutical compositions.

Accordingly, the present invention also provides a pharmaceutical composition comprising a derivative of the invention in combination with a pharmaceutically acceptable carrier.

The compositions according to the invention may be formulated in accordance with routine procedures for administration by any route, such as oral, topical, parenteral, sublingual or transdermal or by inhalation. The compositions may be in the form of tablets, capsules, powders, granules, lozenges, creams or liquid preparations, such as oral or sterile parenteral solutions or suspensions or in the form of a spray, aerosol or other conventional method for inhalation.

The topical formulations of the present invention may be presented as, for instance, ointments, creams or lotions, eye ointments and eye or ear drops, impregnated dressings and aerosols, and may contain appropriate conventional additives such as preservatives, solvents to assist drug penetration and emollients in ointments and creams.

The formulations may also contain compatible conventional carriers, such as cream or ointment bases and ethanol or oleyl alcohol for lotions. Such carriers may be

present as from about 1% up to about 98% of the formulation. More usually they will form up to about 80% of the formulation.

Tablets and capsules for oral administration may be in unit dose presentation form, and may contain conventional excipients such as binding agents, for example syrup, acacia, gelatin, sorbitol, tragacanth, or polyvinylpyrollidone; fillers, for example lactose, sugar, maize-starch, calcium phosphate, sorbitol or glycine; tabletting lubricants, for example magnesium stearate, talc, polyethylene glycol or silica; disintegrants, for example potato starch; or acceptable wetting agents such as sodium lauryl sulphate. Tablets may also contain agents for the stablisation of polypeptide drugs against proteolysis and absorbtion-enhancing agents for macromolecules. The tablets may be coated according to methods well known in normal pharmaceutical practice.

Suppositories will contain conventional suppository bases, e.g. cocoa-butter or other glyceride.

For parenteral administration, fluid unit dosage forms are prepared utilizing the compound and a sterile vehicle, water being preferred. The compound, depending on the vehicle and concentration used, is dissolved in the vehicle. In preparing solutions the compound can be dissolved in water for injection and filter sterilised before filling into a suitable vial or ampoule and sealing.

Parenteral formulations may include sustained-release systems such as encapsulation within microspheres of biodegradable polymers such as poly-lactic co-glycolic acid.

Advantageously, agents such as a local anaesthetic, preservative and buffering agents can be dissolved in the vehicle. To enhance the stability, the composition can be frozen after filling into the vial and the water removed under vacuum. The dry lyophilized powder is then sealed in the vial and an accompanying vial of water for injection may be supplied to reconstitute the liquid prior to use. Advantageously, a surfactant or wetting agent is included in the composition to facilitate uniform distribution of the compound.

Compositions of this invention may also suitably be presented for administration to the respiratory tract as a snuff or an aerosol or solution for a nebulizer, or as a microfine powder for insufflation, alone or in combination with an inert carrier such as lactose. In such a case the particles of active compound suitably have diameters of less than 50 microns, preferably less than 10 microns for example diameters in the range of 1-50 microns, 1-10 microns or 1-5 microns. Where appropriate, small amounts of anti-asthmatics and bronchodilators, for example sympathomimetic amines such as isoprenaline, isoetharine, salbutamol, phenylephrine and ephedrine; xanthine derivatives

such as theophylline and aminophylline and corticosteroids such as prednisolone and adrenal stimulants such as ACTH may be included.

Microfine powder formulations may suitably be administered in an aerosol as a metered dose or by means of a suitable breath-activated device.

Suitable metered dose aerosol formulations comprise conventional propellants, cosolvents, such as ethanol, surfactants such as oleyl alcohol, lubricants such as oleyl alcohol, desiccants such as calcium sulphate and density modifiers such as sodium chloride.

Suitable solutions for a nebulizer are isotonic sterilised solutions, optionally buffered, at for example between pH 4-7, containing up to 20mg ml^{-1} of compound but more generally 0.1 to 10mg ml^{-1}, for use with standard nebulisation equipment.

The quantity of material administered will depend upon the potency of the derivative and the nature of the complaint be decided according to the circumstances by the physician supervising treatment. However, in general, an effective amount of the polypeptide for the treatment of a disease or disorder is in the dose range of 0.01-100mg/kg per day, preferably 0.1mg-10mg/kg per day, administered in up to five doses or by infusion.

No adverse toxicological effects are indicated with the compounds of the invention within the above described dosage range.

The invention also provides a derivative of the invention for use as a medicament.

The invention further provides a method of treatment of disorders amenable to treatment by a soluble peptide which comprises administering a soluble derivative of said soluble peptide according to the invention, and the use of a derivative of the invention for the preparation of a medicament for treatment of such disorders.

In one preferred aspect the present invention relates to derivatives for use in the therapy of disorders involving complement activity and various inflammatory and immune disorders.

In this preferred aspect the soluble polypeptide which is derivatised in accordance with the invention is a soluble complement inhibitor such as a soluble CR1 polypeptide fragment.

Constituting about 10% of the globulins in normal serum, the complement system is composed of many different proteins that are important in the immune system's response to foreign antigens. The complement system becomes activated when its primary components are cleaved and the products alone or with other proteins, activate additional complement proteins resulting in a proteolytic cascade. Activation of the complement system leads to a variety of responses including increased vascular permeability, chemotaxis of phagocytic cells, activation of inflammatory cells,

opsonization of foreign particles, direct killing of cells and tissue damage. Activation of the complement system may be triggered by antigen-antibody complexes (the classical pathway) or, for example, by lipopolysaccharides present in cell walls of pathogenic bacteria (the alternative pathway).

Complement receptor type 1 (CR1) has been shown to be present on the membranes of erythrocytes, monocytes/macrophages, granulocytes, B cells, some T cells, splenic follicular dendritic cells, and glomerular podocytes. CR1 binds to the complement components C3b and C4b and has also been referred to as the C3b/C4b receptor. The structural organisation and primary sequence of one allotype of CR1 is known (Klickstein *et al.*, 1987, J. Exp. Med. 165:1095-1112, Klickstein *et al.*, 1988, J. Exp. Med. 168:1699-1717; Hourcade *et al.*, 1988, J. Exp. Med. 168:1255-1270, WO 89/09220, WO 91/05047). It is composed of 30 short consensus repeats (SCRs) that each contain around 60-70 amino acids. In each SCR, around 29 of the average 65 amino acids are conserved. Each SCR has been proposed to form a three dimensional triple loop structure through disulphide linkages with the third and first and the fourth and second half-cystines in disulphide bonds. CR1 is further arranged as 4 long homologous repeats (LHRs) of 7 SCRs each. Following a leader sequence, the CR1 molecule consists of the N-terminal LHR-A, the next two repeats, LHR-B and LHR-C, and the most C-terminal LHR-D followed by 2 additional SCRs, a 25 residue putative transmembrane region and a 43 residue cytoplasmic tail.

Based on the mature CR1 molecule having a predicted N-terminal glutamine residue, hereinafter designated as residue 1, the first four SCR domains of LHR-A are defined herein as consisting of residues 2-58, 63-120, 125-191 and 197-252, respectively, of mature CR1.

Several soluble fragments of CR1 have been generated via recombinant DNA procedures by eliminating the transmembrane region from the DNAs being expressed (WO 89/09220, WO 91/05047). The soluble CR1 fragments were functionally active, bound C3b and/or C4b and demonstrated Factor I cofactor activity depending upon the regions they contained. Such constructs inhibited *in vitro* complement-related functions such as neutrophil oxidative burst, complement mediated hemolysis, and C3a and C5a production. A particular soluble construct, sCR1/pBSCR1c, also demonstrated *in vivo* activity in a reversed passive Arthus reaction (WO 89/09220, WO 91/05047; Yeh *et al.*, 1991, J. Immunol. 146:250), suppressed post-ischemic myocardial inflammation and necrosis (WO 89/09220, WO 91/05047; Weisman *et al.*, Science, 1990, 249:146-1511; Dupe, R. *et al.* Thrombosis & Haemostasis (1991) 65(6) 695.) and extended survival rates following transplantation (Pruitt & Bollinger, 1991, J. Surg. Res 50:350; Pruitt *et al.*, 1991 Transplantation 52; 868). Furthermore, co-formulation of sCR1/pBSCR1c

with p-anisoylated human plasminogen-streptokinase-activator complex (APSAC) resulted in similar anti-haemolytic activity as sCR1 alone, indicating that the combination of the complement inhibitor sCR1 with a thrombolytic agent was feasible (WO 91/05047).

The soluble CR1 polypeptide fragment encoded by sCR1/pBSCR1c, residues 1-1929 of CR1, may be derivatised in accordance with the invention.

Soluble polypeptides corresponding to part of CR1 have been found to possess functional complement inhibitory, including anti-haemolytic, activity. WO94/00571 discloses soluble polypeptides comprising, in sequence, one to four short consensus repeats (SCR) selected from SCR 1, 2, 3 and 4 of long homologous repeat A (LHR-A) as the only structurally and functionally intact SCR domains of CR1 and including at least SCR3.

In preferred aspects, the polypeptide comprises, in sequence, SCR 1, 2, 3 and 4 of LHR-A or SCR 1, 2 and 3 of LHR-A as the only structurally and functionally intact SCR domains of CR1.

In one aspect, the polypeptides may be represented symbolically as follows:

$$NH_2\text{-}V^1\text{-SCR1-}W^1\text{-SCR2-}X^1\text{-SCR3-}Y^1\text{-OH} \qquad (A)$$

in which SCR1 represents residues 2-58 of mature CR1, SCR2 represents residues 63-120 of mature CR1, SCR3 represents residues 125-191 of mature CR1, and V^1, W^1, X^1 and Y^1 represent bonds or short linking sequences of amino acids, preferably 1 to 5 residues in length and which are preferably derived from native interdomain sequences in CR1.

In a preferred embodiment of formula (I), W^1, X^1 and Y^1 represent residues 59-62, 121-124 and 192-196, respectively, of mature CR1 and V^1 represents residue 1 of mature CR1 optionally linked via its N-terminus to methionine.

In another aspect the polypeptides may be represented symbolically as follows:

$$NH_2\text{-}V^2\text{-SCR1-}W^2\text{-SCR2-}X^2\text{-SCR3-}Y^2\text{-SCR4-}Z^2\text{OH} \qquad (B)$$

in which SCR1, SCR2 and SCR3 are as hereinbefore defined, SCR4 represents residues 197-252 of mature CR1 and V^2, W^2, X^2, Y^2 and Z^2 represents bonds or short linking sequences of amino acids, preferably 1 to 5 residues in length and which are preferably derived from native interdomain sequences in CR1.

In preferred embodiments of formula (II), W^2, X^2, Y^2 and Z^2 represent residues 59-62, 121-124, 192-196, and residues 253 respectively, of mature CR1 and V^2 represents residue 1 of mature CR1 optionally linked via its N-terminus to methionine.

In one particular embodiment of formula (B) arginine 235 is replaced by histidine.

In the preferred embodiment of formula (B), residue 235 is arginine.

In one further aspect, the polypeptide may be represented symbolically as follows:

$$NH_2\text{-}X^3\text{-}SCR3\text{-}Y^3\text{-}OH \qquad (C)$$

in which SCR3 is as hereinbefore defined and X^3 and Y^3 represent bonds or short linking sequences of amino acids, preferably 1 to 5 residues in length and which are preferably derived from native interdomain sequences in CR1.

In a preferred embodiment of formula (C) X^3 represents amino acids 122-124 of mature CR1 optionally linked to methionine at its N-terminus and Y^4 represents amino acids 192-196 of mature CR1.

In another further aspect, the polypeptide may be represented symbolically as follows:

$$NH_2\text{-}X^4\text{-}SCR3\text{-}Y^4\text{-}SCR4\text{-}Z^4\text{-}OH \qquad (D)$$

in which SCR3 and SCR4 are as hereinbefore defined and X^4, Y^4 and Z^4 represent bonds or short linking sequences of amino acids, preferably 1 to 5 residues in length and which are preferably derived from native interdomain sequences in CR1.

In a preferred embodiment of formula (D) X^4 represents amino acids 122-124 of mature CR1 optionally linked to methionine at its N-terminus and Y^4 and Z^4 represent amino acids 192-196 and 253 respectively of mature CR1.

The soluble CR1 polypeptide is derivatised in accordance with the invention by any convenient strategy from those outlined above. In a preferred embodiment the soluble CR1 polypeptide consists of residues 1-196 of CR1 and with an N-terminal methionine and the derivative comprises a myristoyl group and one or more polypeptides sequence selected from

DGPKKKKKKSPSKSSGC

GSSKSPSKKKKKKPGDC

CDGPKKKKKKSPSKSSK

SKDGKKKKKKSKTKC

CSAAPSSGFRILLLKV

AAPSVIGFRILLLKVAGC

and

DGPSEILRGDFSSC

(N-terminus on left).

The soluble complement inhibitor, such as a soluble CR1 polypeptide, derivative of this invention is useful in the treatment of many complement-mediated or complement-related diseases and disorders including, but not limited to, those listed below.

Disease and Disorders Involving Complement

Neurological Disorders

multiple sclerosis

stroke

Guillain Barré Syndrome

traumatic brain injury

Parkinson's disease

allergic encephalitis

Alzheimer's disease

Disorders of Inappropriate or Undesirable Complement Activation

haemodialysis complications

hyperacute allograft rejection

xenograft rejection

corneal graft rejection

interleukin-2 induced toxicity during IL-2 therapy

paroxysmal nocturnal haemoglobinuria

Inflammatory Disorders

inflammation of autoimmune diseases

Crohn's Disease

adult respiratory distress syndrome

thermal injury including burns or frostbite

uveitis

psoriasis

asthma

acute pancreatitis

Post-Ischemic Reperfusion Conditions

myocardial infarction

balloon angioplasty

atherosclerosis (cholesterol-induced) & restenosis

hypertension

post-pump syndrome in cardiopulmonary bypass or renal haemodialysis

renal ischemia

intestinal ischaemia

Infectious Diseases or Sepsis
multiple organ failure
septic shock

Immune Complex Disorders and Autoimmune Diseases
rheumatoid arthritis
systemic lupus erythematosus (SLE)
SLE nephritis
proliferative nephritis
glomerulonephritis
haemolytic anemia
myasthenia gravis

Reproductive Disorders
antibody- or complement-mediated infertility

Wound Healing

The present invention is also directed to a pharmaceutical composition for treating a disease or disorder associated with inflammation or inappropriate complement activation comprising a therapeutically effective amount of a soluble complement inhibitor, such as a soluble CR1 polypeptide, derivative of this invention, and a pharmaceutically acceptable carrier or excipient.

The present invention also provides a method of treating a disease or disorder associated with inflammation or inappropriate complement activation comprising administering to a subject in need of such treatment a therapeutically effective amount of a soluble complement inhibitor, such as a soluble CR1 polypeptide, derivative of this invention.

In the above methods, the subject is preferably a human.

Further provided is the use of a soluble complement inhibitor, such as a soluble CR1 polypeptide, derivative of this invention in the manufacture of a medicament for the treatment of a disease or disorder associated with inflammation or inappropriate complement activation.

In order to inhibit complement activation and, at the same time, provide thrombolytic therapy, the present invention provides compositions which further comprise a therapeutically active amount of a thrombolytic agent. An effective amount of a thrombolytic agent is in the dose range of 0.01-10mg/kg; preferably 0.1-5mg/kg.

Preferred thrombolytic agents include, but are not limited to, streptokinase, human tissue type plasminogen activator and urokinase molecules and derivatives, fragments or conjugates thereof. The thrombolytic agents may comprise one or more chains that may be fused or reversibly linked to other agents to form hybrid molecules (EP-A-0297882 and EP 155387), such as, for example, urokinase linked to plasmin (EP-A-0152736), a fibrinolytic enzyme linked to a water-soluble polymer (EP-A-0183503). The thrombolytic agents may also comprise muteins of plasminogen activators (EP-A-0207589). In a preferred embodiment, the thrombolytic agent may comprise a reversibly blocked *in vitro* fibrinolytic enzyme as described in U.S. Patent No. 4,285,932. A most preferred enzyme is the p-anisoyl plasminogen-streptokinase activator complex, anistreplase as described in U.S. Patent No. 4,808,405 (Monk *et al.*, 1987, Drugs 34:25-49).

Routes of administration for the individual or combined therapeutic compositions of the present invention include standard routes, such as, for example, intravenous infusion or bolus injection. Active complement inhibitors and thrombolytic agents may be administered together or sequentially, in any order.

The present invention also provides a method for treating a thrombotic condition, in particular acute myocardial infarction, in a human or non-human animal. This method comprises administering to a human or animal in need of this treatment an effective amount of a soluble complement inhibitor, such as a soluble CR1 polypeptide, derivative according to this invention and an effective amount of a thrombolytic agent.

Also provided is the use of a soluble complement inhibitor, such as a soluble CR1 polypeptide, derivative of this invention and a thrombolytic agent in the manufacture of a medicament for the treatment of a thrombotic condition in a human or animal. Such methods and uses may be carried out as described in WO 91/05047.

This invention further provides a method for treating adult respiratory distress syndrome (ARDS) in a human or non-human animal. This method comprises administering to the patient an effective amount of a soluble complement inhibitor, such as a soluble CR1 polypeptide, derivative according to this invention.

The invention also provides a method of delaying hyperacute allograft or hyperacute xenograft rejection in a human or non-human animal which receives a transplant by administering an effective amount of a soluble complement inhibitor, such as a soluble CR1 polypeptide, derivative according to this invention. Such administration may be to the patient or by application to the transplant prior to implantation.

The invention yet further provides a method of treating wounds in a human or non-human animal by administering by either topical or parenteral e.g. intravenous

routes, an effective amount of a soluble complement inhibitor, such as a soluble CR1 polypeptide derivative according to this invention.

In another preferred aspect the soluble polypeptide is a thrombolytic agent such as prourokinase, streptokinase, tissue-type plasminogen activator or reteplase and the derivative of the invention is useful in the treatment of thrombotic disorders such as acute myocardial infarction. The invention thus provides a pharmaceutical composition for treating thrombotic disorders comprising a therapeutically effective amount of a derivative of a thrombolytic agent according to the invention, and a pharmaceutically acceptable carrier or excipient. The invention further provides a method of treatment of thrombotic disorders by administering an effective amount of a derivative of a thrombolytic agent according to the invention, and the use of such derivative in the preparation of a medicament for the treatment of thrombotic disorders.

The following Methods and Examples illustrate the invention.

GENERAL METHODS USED IN EXAMPLES

(i) DNA Cleavage

Cleavage of DNA by restriction endonucleases was carried out according to the manufacturer's instructions using supplied buffers. Double digests were carried out simultaneously if the buffer conditions were suitable for both enzymes. Otherwise double digests were carried out sequentially where the enzyme requiring the lowest salt condition was added first to the digest. Once the digest was complete the salt concentration was altered and the second enzyme added.

(ii) DNA ligation

Ligations were carried out using T4 DNA ligase purchased from Promega, as described in Sambrook *et al*, (1989) Molecular Cloning: A Laboratory Manual 2nd Edition. Cold Spring Harbour Laboratory Press.

(iii) Plasmid isolation

Plasmid isolation was carried out by the alkaline lysis method described in Sambrook *et al*, (1989) Molecular Cloning: A Laboratory Manual 2nd Edition. Cold Spring Harbour Laboratory Press or by one of two commercially available kits: the Promega Wizard™ *Plus* Minipreps or Qiagen Plasmid Maxi kit according to the manufacturer's instructions.

(iv) DNA fragment isolation

DNA fragments were excised from agarose gels and DNA extracted using one of three commercially available kits: the QIAEX gel extraction kit or Qiaquick gel extraction kit (QIAGEN Inc., USA), or GeneClean (Bio 101 Inc, USA) according to the manufacturer's instructions.

(v) Introduction of DNA into *E. coli*

Plasmids were transformed into *E. coli* BL21(DE3) (Studier and Moffat, (1986), *J. Mol. Biol* 189:113), *E. coli* XLI-blue, BL21 (DE3) pLys-S or BLR (DE3) pLys-S that had been made competent using calcium chloride as described in Sambrook *et al*, (1989). Cell lines were purchased as frozen competent cultures from Stratagene. *E. coli* JM109 was purchased as a frozen competent culture from Promega.

(vi) DNA sequencing

Plasmid DNA was sequenced on a Vistra DNA Labstation 625. The sequencing chemistry was performed using Amersham International's 'Thermo Sequenase fluorescent dye-terminator cycle sequencing kit' (RPN 2435), in conjunction with their 'FMP fluorescent dye-terminator precipitation kit' (RPN 2433) according to the manufacturer's instructions.

The sequences produced by the above procedure were analysed by a Perkin Elmer ABI Prism 377 DNA Sequencer. This is an electrophoretic technique using 36 cm x 0.2mm 4% acrylamide gels, the fluorescently labeled DNA fragments being detected by a charge coupled device camera according to the manufacturer's instructions.

(vii) Production of oligonucleotides

Oligonucleotides were purchased from Cruachem.

(viii) pBROC413

The plasmid pT7-7 [Tabor, S (1990), Current Protocols in Molecular Biology, F. A. Ausubel, Brent, R. E. Kingston, D. D. Moore, J. G. Seidman, J. A. Smith, and K. Struhl,eds.] pp.16.2.1-16.2.11, Greene Publishing and Wiley-Interscience,New York.] contains DNA corresponding to nucleotides 2065-4362 of pBR322 and like pBR322 can be mobilized by a conjugative plasmid in the presence of a third plasmid ColK. A mobility protein encoded by ColK acts on the *nic* site at nucleotide 2254 of pBR322 initiating mobilization from this point. pT7-7 was digested with *Lsp*I and *Bgl*II and the protruding 5' ends filled in with the Klenow fragment of DNA PolymeraseI. The plasmid DNA fragment was purified by agarose gel electrophoresis, the blunt ends ligated together and transformed into *E. coli* DH1 by electroporation using a Bio-Rad Gene Pulser and following the manufacturers recommended conditions. The resultant plasmid pBROC413 was identified by restriction enzyme analysis of plasmid DNA.

The deletion in pBROC413 from the *Lsp*I site immediately upstream of the f 10 promoter to the *Bgl*II site at nucleotide 434 of pT7-7 deletes the DNA corresponding to nucleotides 2065-2297 of pBR322. The *nic* site and adjacent sequences are therefore deleted making pBROC413 non mobilizable.

(ix) Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS PAGE)

SDS PAGE was carried out generally using the Novex system (British Biotechnology) according to the manufacturer's instructions. Prepacked gels of 4 - 20% acrylamide were used. Samples for electrophoresis, including protein molecular weight standards (for example LMW Kit, Pharmacia or Novex Mark 12) were usually diluted in

1%(w/v)SDS - containing buffer (with or without 5%(v/v) 2-mercaptoethanol), and left at room temperature for about 10 to 30 min before application to the gel.

(x) Reduction of disulphides and modification of thiols in proteins

There are a number of methods used for achieving the title goals. The reason it may be necessary to carry out selective reduction of disulphides is that during the isolation and purification of multi-thiol proteins, in particular during refolding of fully denatured multi-thiol proteins, inappropriate disulphide pairing can occur. In addition, even if correct disulphide paring does occur, it is possible that a free cysteine in the protein may become blocked, for example with glutathione. These derivatives are generally quite stable. In order to make them more reactive, for example for subsequent conjugation to another functional group, they need to be selectively reduced, with for example dithiothreitol (DTT) or Tris (2-carboxyethyl) phosphine.HCl (TCEP) then optionally modified with a function which is moderately unstable. An example of the latter is Ellmans reagent (DTNB) which gives a mixed disulphide. In the case where treatment with DTNB is omitted, careful attention to experimental design is necessary to ensure that dimerisation of the free thiol-containing protein is minimised. Reference to the term 'selectively reduced' above means that reaction conditions eg. duration, temperature, molar ratios of reactants have to be carefully controlled so that reduction of disulphide bridges within the natural architecture of the protein is minimised. All the reagents are commercially available eg. from Sigma or Pierce.

The following general examples illustrate the type of conditions that may be used and that are useful for the generation of free thiols and their optional modification. The specific reaction conditions to achieve optimal thiol reduction and/or modification are ideally determined for each protein batch.

TCEP may be prepared as a 20mM solution in 50mM Hepes (approx. pH 4.5) and may be stored at -40 degrees C. DTT may be prepared at 10mM in sodium phosphate pH 7.0 and may be stored at -40 degrees C. DTNB may be prepared at 10mM in sodium phosphate pH 7.0 and may be stored at -40 degrees C. All of the above reagents are typically used at molar equivalence or molar excess over protein concentration, the precise concentrations ideally identified experimentally. The duration and the temperature of the reaction are similarly determined experimentally. Generally the duration would be in the range 1 to 24 hours and the temperature would be in the range 2 to 30 degrees C. Excess reagent may be conveniently removed by buffer exchange, for example using Sephadex G25 or Sephadex G50. A suitable buffer is 0.1M sodium phosphate pH7.0 or the solution may be left untreated.

EXAMPLES

Example 1 Preparation of N-(Myristoyl) 2-aminoethane thiol (MAET)

Myristoyl chloride (1.0 mmol)was added with vigorous stirring to ice-cooled dry pyridine (1.0ml), and followed immediately by N-hydroxysuccinimide (1.5 mmol).The mixture was stirred for 4h at ambient termperature (~23°C). 2-aminoethanethiol free base (1.1 mmol) was added as solid to the mixture and allowed to react for 6h at ambient temperature, followed by 3 days at 4°C. The product was treated with water (5ml), stirred for 1h at ambient and filtered, washing with cold water. The white solid was dissolved in dimethylsulphoxide and reprecipitated with water and then vacuum dried over phosphorous pentoxide. The final yield was 0.21g (~70%). Thiol titration using Ellman's reagent indicated that the product contained ~45% free thiol.

Example 2 Synthesis of Myristoyl/Electrostatic Switch Peptide Reagent 1 (MSWP-1) (SEQ ID NO: 27)

N-(Myristoyl) -Gly-Ser-Ser-Lys-Ser-Pro-Ser-Lys-Lys-Lys-Lys-Lys-Lys-Pro-Gly-Asp-(S-2-Thiopyridyl)Cys-NH_2

The peptide:
Gly-Ser-Ser-Lys-Ser-Pro-Ser-Lys-Lys-Lys-Lys-Lys-Lys-Pro-Gly-Asp-Cys-NH_2
(SEQ ID NO: 5)
was prepared using solid phase synthesis via the general Fmoc/tBu strategy developed by Sheppard and Atherton (E.Atherton and R.C.Sheppard, Solid Phase Synthesis, IRL Press, Oxford, 1989). Kieselguhr-supported polydimethylacrylamide resin (Macrosorb 100) was used as the solid support and was derivatised with ethylene diamine.

Coupling reactions were carried out using N-α-Fmoc protected reagents pre-activated with N,N'-diisopropylcarbodiimide/ N-hydroxybenzotriazole (in 4-fold molar excess) with bromophenol blue monitoring. Fmoc cleavages used 20% piperidine in DMF. Reactions to assemble the peptide chain were carried out by repeated cycles of coupling and deprotection including the attachment of the modified Rink linkage reagent (p-[(R,S)-α-[1-(9H-fluoreny-9-yl-methoxyformamido] 2,4 dimethoxybenzyl]-phenoxyacetic acid) designed to yield a C-terminal amide on final cleavage. The side chain functionalities of the individual amino-acids were protected as follows:

Ser (tButyl), Lys (Boc), Asp (O-tButyl), Cys (Trityl).

On completion of the peptide assembly and with the peptide still attached to the resin, the myristoyl group was attached to the amino group of the N terminal glycine by direct coupling of myristic acid by the same activation procedure. This modified peptide was then cleaved from the resin and the side-chain protecting groups removed at the

same time by treatment with trifluoracetic acid containing 2.5% water and 2.5% triisopropyl silane.

The crude product was treated with 2,2' dithiopyridine in 0.01M ammonium acetate solution at pH 8-9 for approx. 2h, then acidified with acetic acid and purified by preparative high performance liquid chromatography (HPLC) in 0.1% trifluoracetic acid (TFA) /water and 0.1% TFA/acetonitrile as gradient component. After lyophilisation, the peptide was a white amorphous powder, soluble to at least 10mg/ml in dimethylsulphoxide. Fast atom bombardment mass spectrometry gave main peaks at m/e 2107.8, 2129.7 and 2145.8, corresponding to the monoprotonated, monosodiated and monopotassiated molecular ions of the peptide. The 2-thiopyridyl content of the peptide was measured by dissolving it to around 0.03mM to 0.2 mM in 0.1M Sodium Borate pH 8.0 and reducing by addition of dithiothreitol to 5mM. The change in optical density at 343nm was used to calculate the amount of pyridine 2-thione released using an extinction coefficient at this wavelength of 8080 cm^{-1} M^{-1} . This indicated that the peptide content was approximately 60% of the dry weight.

Example 3 Synthesis of Myristoyl/Electrostatic Switch Peptide Reagent 2 (MSWP-2) (SEQ ID NO: 28)

N-acetyl-Cys (2-thiopyridyl) Asp-Gly-Pro-Lys-Lys-Lys-Lys-Lys-Lys-Ser-Pro-Ser Lys-Ser-Ser-Lys-(ε-N-(Myristoyl))-NH_2

The peptide:
Cys-Asp-Gly-Pro-Lys-Lys-Lys-Lys-Lys-Lys-Ser-Pro-Ser-Lys-Ser-Ser-Lys-NH_2
(SEQ ID NO: 18) was prepared by solid-phase synthesis using the general method described in Example 2 and with the following variations:

a. The C-terminal lysine was protected by alkylation with the 4-methyl trityl (MTT) group; all other lysines were N-ε protected with the t-Boc group

b. MTT was removed with 1% v/v trifluoracetic acid in dichloromethane and the resulting unique free amino group derivatised with myristic acid prior to deprotection of the other lysines (as described in Example 2)

The N-terminus was acetylated with acetic anhydride upon completion of the peptide chain assembly. Generation of the 2-pyridyldithiocysteine moiety was by reation of the deprotected peptide with 2,2'-dithiopyridine as described above. The product was purified as described in Example 2. Fast-atom bombardment mass spectrometry gave a molecular ion peak at 2221.3 (cf 2220.3 for the monoprotonated theoretical mass).

Amino-acid Analysis:

	Asx	Ser	Gly	Pro
Theory:	1.0	4.0	1.0	2.0
Found	0.97	3.53	1.15	1.88

(Asx = Asn or Asp)

Amino-acid analysis indicated a net peptide content by weight of 68.7%. The 2-pyridyl disulphide content was appoximately 60% by weight using the method of Example 2.

Example 4 Synthesis of Myristoyl/Electrostatic Switch Peptide Reagent 3 (MSWP-3) (SEQ ID NO: 29)

N-(Myristoyl) -Ser-Lys-Asp-Gly-Lys-Lys-Lys-Lys-Lys-Lys-Ser-Lys-Thr-Lys-(S-2-Thiopyridyl)Cys-NH_2

The peptide:

Ser-Lys-Asp-Gly-Lys-Lys-Lys-Lys-Lys-Lys-Ser -Lys-Thr-Lys-Cys (SEQ ID NO: 19) was prepared using the general solid-phase synthesis protocol of Example 2. Myristoylation, C-terminal amidation and derivatisation of the Cys residue were performed as described in Example 2. After purification, mass spectrometry gave the major peak at 2040.5, corresponding to a monoprotonated form (Theory: 2039.5)

Amino-acid analysis:

	Asx	Ser	Gly	Thr	Lys
Theory:	1	2	1	1	9
Found:	1.02	2.04	1.14	1.06	8.85

The peptide content was about 56% by weight

Example 5 Synthesis of T-Cell Targeting Peptide Reagent 1 (TCTP-1) (SEQ ID NO: 30)

N-acetyl-(2-thiopyridyl)Cys Ser-Ala-Ala-Pro-Ser-Ser-Gly-Phe-Arg-Ile-Leu-Leu-Leu-Lys-Val-$CONH(CH_2)_9CH_3$

The peptide Cys-Ser-Ala-Ala-Pro-Ser-Ser-Gly-Phe-Arg-Ile-Leu-Leu-Leu-Lys-Val (SEQ ID NO: 20) was prepared using the general solid-phase methodology of Example 2 and N-acetylated as in Example 3. The C-terminus was derivatised using n-decylamine in place of the Rink reagent. Mass spectrometry of the purified peptide gave a major peak at 1952.3 corresponding to a monoprotonated molecular ion (Theory:

1951.1.) An ion at 1843.3 was also observed , this is believed to correspond to loss of the thiopyridyl group in the spectrophotometer.

Amino-acid analysis:

	Ser	Gly	Arg	Ala	Pro	Val	Ile	Phe	Leu	Lys
Theory:	3	1	1	2	1	1	1	1	3	1
Found:	2.95	1.10	1.10	2.11	1.04	0.60	0.92	1.00	3.03	1.03

The peptide content by weight was 53%

Example 6 Expression and isolation of [SCR1-3]-Cys (SEQ ID NO: 6)

(a) Construction of plasmid pDB1030 encoding [SCR 1-3]-Cys

The plasmid coding for SCR1-3 of LHR-A of CR1, pDB1013-5 (patent application WO 94/00571) was digested with restriction endonucleases *Eco*RI and *Hin*dIII and the 2.2 kB plasmid band was isolated from an agarose gel using a Qiagen Qiaex DNA extraction kit according to the manufacturer's instructions. This is fragment 1. A second batch of pDB1013-5 was digested with *Ban*I and *Eco*RI and the 196 bp band was extracted from agarose as above. This is fragment 2. Two oligonucleotides, SEQ ID No.1 and SEQ ID No.2, were annealed to give a final DNA concentration of 100 pmoles/ul. The annealed oligo has a *BanI/Eco*RI overhang and duplicates the sequence at the 3' end of pDB1013-5 but in addition contains a codon coding for cysteine just before the stop codon. This is fragment 3.

Fragments 1,2 and 3 were ligated with T4 DNA ligase in a single reaction to give pDB1030 . The ligated plasmid was transformed into competent *E. coli* JM109 purchased from Promega. Resulting colonies were analysed by restriction endonuclease digestion and DNA sequencing confirmed that the encoded amino acid sequence of SCR(1-3) (SEQ ID No.27 of WO 94/00571) had been altered by a single C-terminal cysteine residue to give SEQ ID No.6.

(b) Expression of [SCR1-3]-Cys from pDB1030

pDB1030 was transformed into calcium chloride competent *E. coli* BL21(DE3) and resultant colonies were isolated and checked for plasmid content. To express protein from pDB1030 in *E. coli* BL21(DE3), a single colony was inoculated into 10 ml LB-phosphate media (20g/L tryptone, 15g/L yeast extract, 0.8g/L NaCl, 0.2g/L Na_2HPO_4, 0.1g/L KH_2PO_4) containing 50ug/ml ampicillin. The culture was grown for 6 hours at 37°C, 230 r.p.m. before being used to inoculate 100 ml of the same media containing 50 ug/ml ampicillin. Growth was under the same conditions overnight. 25 ml of each

culture were then used to inoculate 600 ml of the same media with 50 ug/ml ampicillin in 3 L erlenmeyer flasks. Cells were grown to an OD of 0.8 - 1.0 at A_{600} nm. IPTG (isopropyl B-D galactopyranoside) was added to a final concentration of 1 mM and cells allowed to continue growth for a further 3 - 4 hours before harvesting by centrifugation at 8000 g/10 min. Pellet from 2L of culture was stored at -80°C

(c) Isolation, refolding, purification and formulation of [SCR1-3]-Cys

The methods described are essentially those detailed in Dodd I. et al (1995) Protein Expression and Purification 6 727-736.

i) Isolation of solubilised inclusion bodies

The frozen cell pellet of *E. coli* BL21(DE3) (pDB1030) was resuspended in 50 mM Tris/50 mM NaCl/1 mM EDTA/0.1 mM PMSF pH 8.0 at a ratio of 33 ml for each litre of culture pellet. The suspension was transferred to a glass beaker surrounded by ice and sonicated (Heat systems - Ultrasonics W380; 50 x 50% pulse, pulse time = 5 sec.) for typically 3 - 6 minutes. The disrupted pellet was then frozen and stored at -80°C. Approx. 2 weeks later the sonicate was thawed and centrifuged at approx. 8000g for 20 min. The pellet was resuspended in 20 mM Tris/8M urea/1 mM EDTA/50 mM 2-mercaptoethanol pH 8.5 (200ml) at room temperature by vigorous swirling, then left for 1h at room temperature followed by overnight at 4° C.

ii) Initial purification using SP-Sepharose

To the viscous solution was added SP-Sepharose FF (approx. 30g wet weight) that had been water washed and suction-dried. The mixture was swirled vigorously and left static for 1-2h at room temperature. The supernatant was decanted, sampled and discarded. The remaining slurry was resuspended to a uniform suspension and poured into a glass jacket and allowed to settle into a packed bed. The column was equilibrated with 0.02M Tris/8M urea/0.05M 2-mercaptoethanol/0.001 M EDTA pH 8.5.at 4°C. When the A_{280} of the eluate had stabilised at baseline, the buffer was changed to equilibration buffer additionally containing 1M NaCl. A single A_{280} peak was eluted by the 1M NaCl-containing buffer; the volume was approx. 50ml. The protein concentration of the solution was estimated by A_{280} determination, using a molar extinction coefficient of 25000 cm^{-1} of a sample that had been buffer-exchanged (Sephadex G25) into 50 mM formic acid. This showed the product had a protein concentration of 1.6mg/ml. The solution was stored at -40°C.

iii) Folding and further processing

25ml of the SP-Sepharose-purified product was added gradually over a 1 min period to 780 ml freshly prepared, cold 0.02M ethanolamine/1mM EDTA with continuous swirling, and left static for 1 h/4°C. Reduced glutathione (GSH) was added

to 1 mM and oxidised glutathione (GSSG) was added to 0.5 mM. The solution was clear and was left static approx 2-3°C for 3 d. The solution was then ultrafiltered using a YM10 membrane to a final retentate volume of about 35 ml; the retentate was slightly cloudy and had the appearance of a translucent solution. It was stored for 12 days at 4° C. It was then spun at 30 000g for 15 mins and the supernatant mixed with 9 vol. 0.1M NaH_2PO_4/1M $(NH_4)_2S0_4$ pH 7.0 (Buffer A) at room temperature and immediately centrifuged at 3000 rpm for 15 min. The supernatant was ultrafiltered (YM10) to about 4ml and then buffer-exchanged into 0.1M sodium phosphate pH 7.0 (5.0ml); this solution had a protein concentration of 1.7mg/ml by A280 analysis. It was treated with dithio bis nitrobenzoic acid (DTNB) (8-fold molar excess) for 30 min at room temperature. Free thiol content based on A412 measurement and an extinction coefficient (for the free thionitrobenzoate ion) of 13 600 was 6uM equivalent to only about 10% derivatisation to give Product A. The majority of the product was believed to be [SCR1-3]-Cys where the free C-terminal thiol was blocked by reaction with glutathione or 2-mercaptoethanol during the refolding stage.

(d) Alternative method for isolation, refolding, purification and formulation of [SCR1-3]-Cys

The method was similar to that described above, except that it more closely followed the procedures described in Dodd et al (op cit.). Notably, the ultrafiltered retentate post refolding was immediately treated with ammonium sulphate followed by clarification by centrifugation and Butyl Toyopearl chromatography. The resulting A280-absorbing fractions that eluted at about 0.2 to 0.4M ammonium sulphate were pooled and regarded as Product B. Starting with a nominal 100mg of fully reduced SCR1-3/cys, Product B contained 17mg. The product contained one major species by non-reduced SDS PAGE with an estimated purity of >90% and an apparent molecular weight of 21 000. On the basis of studies with similarly produced preparations it was believed to be the S-glutathione and/or S-mercaptoethanol derivatised form of the parent protein, although at least some batches produced in a similar way or stored for a period of time might exist as the free cysteine variant. The product also contained a polypeptide with an apparent molecular weight of about 40 000 On the basis of studies with similar batches of protein enriched in this species it was identified as the dimer of [SCR1-3]-Cys.

Example 7 Expression and isolation of SCR1-3/switch fusion (SEQ ID NO: 7)

H_2N-[SCR 1-3]-Asp-Gly-Pro-Lys-Lys-Lys-Lys-Lys-Lys-Ser-Pro-Ser-Lys-Ser-Ser-Gly-Cys-OH

(a) Construction of plasmid pDB1031 encoding SCR1-3/switch

Fragment 1 and fragment 2 of pDB1013-5 were the same as Example 6 above. Two oligonucleotides, SEQ ID No.3 and SEQ ID No. 4, prepared by Cruachem were annealed to give a final DNA concentration of 100 pmoles/ul. The annealed oligo has an *BanI/Eco*RI overhang and duplicates the sequence at the 3' end of pDB1013-5 but in addition contains 17 additional codons coding for DGPKKKKKKSPSKSSGC just before the stop codon. This is fragment 4.

Fragments 1, 2 and 4 were ligated with T4 DNA ligase in a single reaction to give pDB1031. The ligated plasmid was transfomed into competent *E. coli* JM109. Resulting colonies were analysed by restriction endonuclease digestion and DNA sequencing confirmed that the encoded amino acid sequence of SCR1-3 (SEQ ID 27 of WO 94/00571) had been altered by C terminal addition of amino acids DGPKKKKKKSPSKSSGC to give SEQ ID NO: 7.

(b) Expression of SCR1-3/switch from pDB1031

pDB1031 was transformed into calcium chloride competent *E. coli* BL21(DE3) and resultant colonies were isolated and checked for plasmid content. To express protein from pDB1031 in *E. coli* BL21(DE3), a single colony was inoculated into 10 ml LB-phosphate media (20g/L tryptone, 15g/L yeast extract, 0.8g/L NaCl, 0.2g/L Na_2HPO_4, 0.1g/L KH_2PO_4) containing 50ug/ml ampicillin. The culture was grown for 6 hours at 37°C, 230 r.p.m. before being used to inoculate 100 ml of the same media containing 50 ug/ml ampicillin. Growth was under the same conditions overnight. 25 ml of each culture were then used to inoculate 600 ml of the same media with 50 ug/ml ampicillin in 3 L erlenmeyer flasks. Cells were grown to an OD of 0.8 - 1.0 at A_{600} nm. IPTG (isopropyl B-D galactopyranoside) was added to a final concentration of 1 mM and cells allowed to continue growth for a further 3 - 4 hours before harvesting by centrifugation at 8000 g/10 min. The cell pellet was frozen at -40 degrees C.

(c) Isolation, refolding, purification and formulation of SCR1-3/switch

The methods described are essentially those detailed in Dodd I. et al (1995) Protein Expression and Purification 6 727-736, with some modifications.

i) Isolation of solubilised inclusion bodies

The frozen cell pellet of *E. coli* BL21(DE3) (pDB1031) was thawed and resuspended in 50 mM Tris/50 mM NaCl/1 mM EDTA/0.1 mM PMSF pH 8.0 at a ratio of 33 ml for each litre of culture pellet. The suspension was transferred to a glass beaker surrounded by ice and sonicated (Heat systems - Ultrasonics W380; 50 x 50% pulse, pulse time = 5 sec.) for typically 3 - 6 minutes. The disrupted pellet was then frozen and

stored at -80°C. Approx. 1d later the sonicate was thawed and centrifuged at approx. 8000g for 20 min. The pellet was resuspended in 20 mM Tris/8M urea/1 mM EDTA/50 mM 2-mercaptoethanol pH 8.5 (240ml) at room temperature by vigorous swirling, then left for 1h at room temperature followed by 5 days at 4° C.

ii) **Preliminary purification using SP-Sepharose**

To the viscous solution was added SP-Sepharose FF (approx. 30g wet weight) that had been water washed and suction dried. The mixture was swirled vigorously and left static for approx. 2h at room temperature. The supernatant was decanted, sampled and discarded. The remaining slurry was resuspended to a uniform suspension and poured into a glass jacket and allowed to settle into a packed bed. The column was equilibrated with 0.02M Tris/8M urea/0.05M 2-mercaptoethanol/0.001 M EDTA pH 8.5.at 4°C. When the A_{280} of the eluate had stabilised at baseline, the buffer was changed to equilibration buffer additionally containing 1M NaCl. A single A_{280} peak was eluted by the 1M NaCl-containing buffer; the volume was approx. 50ml. The protein concentration of the solution was estimated by A_{280} determination, using a molar extinction coefficient of 25000 cm^{-1},of a sample that had been buffer-exchanged (Sephadex G25) into 50 mM formic acid. This showed the product had a protein concentration of 2.8mg/ml. Analysis by SDS PAGE/stain showed a major band (approx 80%) at about 23 000Da. The solution was stored at -40°C.

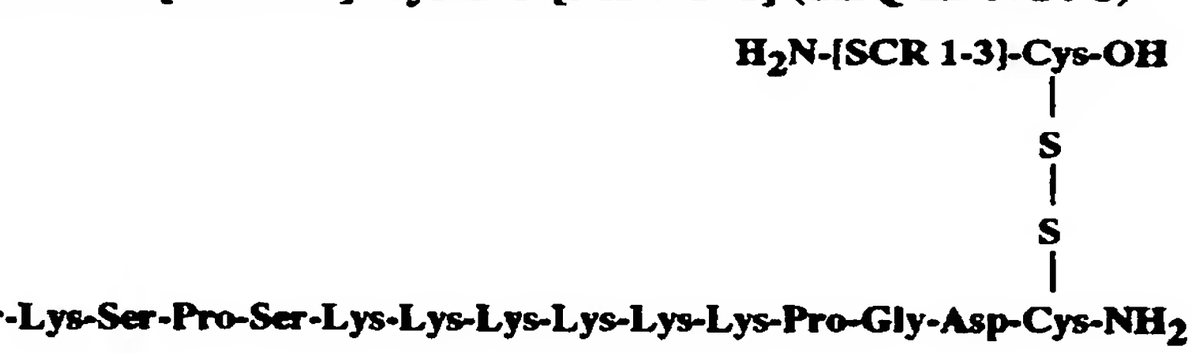
iii) **Folding and further processing**

14ml of the SP-Sepharose-purified product was added gradually over a 1 min period to 430 ml freshly prepared, cold 0.05M Hepes/2 M sodium chloride/1mM EDTA pH 8.0 with continuous swirling, and left static for 1 h/4°C. Reduced glutathione (GSH) was added to 1 mM and oxidised glutathione (GSSG) was added to 0.5 mM. The solution was clear and was left static approx 2-3°C for 3 d. The solution was then ultrafiltered using a YM10 membrane to a final retentate volume of about 34 ml; the retentate was slightly cloudy. It was then spun at 25 000g for 15 mins and the supernatant buffer-exchanged into 0.1M sodium phosphate pH 7.0 (46 ml). This fraction contained 2 mg of protein on the basis of an A280 determination. The solution was mixed with DTNB (20mM; 0.65ml) for 20 min at 4 degrees C and then ultrafiltered to 2.4ml. This retentate was buffer-exchanged into 0.1M sodium phosphate pH 7.0 (3.0ml) and stored at -40 degrees C. Absorbance measurements at 412nm on the solution prior to ultrafiltration suggested 25% derivatisation with DTNB.

(d) Alternative isolation, refolding, purification and formulation of SCR1-3/switch

The method was similar to that described in (c) above, except that following the ultrafiltration step after refolding it more closely followed the procedures described in Dodd et al (*op cit.*). Notably, the ultrafiltered retentate post refolding was immediately treated with ammonium sulphate followed by clarification by centrifugation and Butyl Sepharose chromatography. The resulting A280-absorbing fractions that eluted at about 0.2 to 0.4M ammonium sulphate were pooled and regarded as initial product. Additional treatment with TCEP essentially as above, followed by DTNB yielded a final product at 10uM final protein concentration. The final product contained one major species by non-reduced SDS PAGE with an estimated purity of >90% and an apparent molecular weight of 23 000 and contained about 2 moles TNB per mole of protein.

Example 8 Preparation of [SCR1-3]-Cys-S-S-[MSWP-1] (SEQ ID NO: 8)



(a) Product A of Example 6(c) (1.5ml) was treated with dithiothreitol (30 ul of 0.5M in water, final concentration 10mM) for 60 min at 4°C to give the free peptide SEQ ID NO 6. The yellow solution was gel filtered at 4°C on a small column of Sephadex G-25 (PD-10, Pharmacia) into 0.05M Hepes.HCl buffer pH 7.5 (3.0ml). The slightly cloudy solution was mixed with a solution of MSWP-1 (Example 2) (3.8mM dithiopyridyl equivalents, 150 ul) to a final concentration of 0.18mM (~8 molar equivalents). The mixture was held for 2h on ice and then gel filtered as before but using 2 PD10 columns (1.6 ml applied, 3.2ml eluted). The final eluate was not cloudy and was stored frozen at -70°C in aliquots of 0.4ml.

(b) [SCR1-3]-Cys protein product B described in Example 6(d) (1.5ml; 31uM protein) was mixed with TCEP (20mM; 0.007ml) and incubated at room temperature for 23 h to give the free protein SEQ ID NO: 6. MSWP-1 (Example 2) (10mM; 0.093ml) was added and the solution incubated for a further 4 h. 0.75ml of the final solution was buffer-exchanged into 50mM formic acid and aliquots left in solution or lyophilised. The product was >80% pure by SDS PAGE and had an apparent molecular weight of 23 000, clearly shifted from the original parent molecular weight of 21 000. The

lyophilisate was easily soluble in 50mM formic acid at an estimated protein concentration of 2 mg/ml.

(c) [SCR1-3]-Cys protein product B described in Example 6(d) (21.6ml; 31uM protein) was mixed with TCEP (20mM; 0.1ml) and incubated at room temperature for 22h to give the free protein SEQ ID NO 6. MSWP-1 (20mM in 0.1M sodium phosphate pH 7.0; 0.67ml) was added and the solution incubated for a further 4 h. All 22ml was buffer-exchanged into 50mM formic acid using Sephadex G50 (Vt 160ml). Three A280 peaks were obtained. The first one, eluting at volume 56 -106ml, was the title compound according to SDS PAGE analysis. The fraction was aliquoted and aliquots stored at - 40 degrees C or lyophilised. Amino acid analysis of the pre-lyophilisation solution indicated a protein concentration of 0.42mg/ml. A280 (1cm path length) was 0.44. C8 reverse phase HPLC and SDS PAGE both indicated a purity of approx 80%. The latter technique showed the major band had an apparent molecular weight of 23 000, clearly shifted from the original parent molecular weight of 21 000; on reduction the 23 000 band shifted to two bands with molecular weights of approx 21 000 and approx 5 000. The lyophilisate was easily soluble in 50mM formic acid or in PBS 'A' (Dulbecco) at a protein concentration of 6mg/ml.

(d) [SCR1-3]-Cys-S-S-[MSWP-1] from (c) was divided into 0.3 ml aliquots and freeze-dried. Individual aliquots were resolubilised in 50mM formic acid (0.3ml or 0.039ml).

Example 9 Preparation of [SCR1-3/switch fusion]disulphide linked to [MAET] (SEQ ID NO: 31)

H_2N-[SCR 1-3]-Asp-Gly-Pro-Lys-Lys-Lys-Lys-Lys-Lys-Ser-Pro-Ser-Lys-Ser-Ser-Gly-Cys-OH

$CH_3(CH_2)_{12}CONH-(CH_2)_2-S-S$

Title compound can be synthesised using TNB-activated SCR1-3/switch (SEQ ID NO: 7) prepared as in Example 7(d). The TNB-activated SCR1-3/switch is mixed with a molar excess of MAET (Example 1), which might be typically made up at 2.0mg/ml in DMSO, equivalent to about 3mM free thiol. Typical reaction conditions would be 1 to 4 hours at room temperature or overnight at 4 degrees C using a protein concentration of 1 to 100 uM. The reaction may be monitored by checking the generation of yellow colour, which is caused by the release of free TNB ion. Once the reaction is complete the solution may be buffer exchanged into a suitable buffer, for example 0.1M sodium phosphate pH 7.0, and stored at - 40 degrees C until required.

Example 10 Preparation of [SCR1-3/switch fusion] disulphide linked to [MSWP_1] (SEQ ID NO: 9)

H_2N-[SCR 1-3]-Asp-Gly-Pro-Lys-Lys-Lys-Lys-Lys-Lys-Ser-Pro-Ser-Lys-Ser-Ser-Gly-Cys-OH

S

S

N-(Myristoyl)-Gly-Ser-Ser-Lys-Ser-Pro-Ser-Lys-Lys-Lys-Lys-Lys-Lys-Pro-Gly-Asp-Cys-NH_2

Method (a)

0.02ml of MSWP-1 (Example 2, 10mM in 0.1M sodium phosphate pH 7.0) was mixed with 0.005ml of TCEP (20mM in 50mM Hepes) and left for 10 min at room temperature. The resultant solution was Solution A containing the myristoylated peptide of SEQ ID NO: 5. TNB-activated SCR1-3/switch (SEQ ID NO: 7) prepared in a similar way to that described in Example 7(c) (0.3ml; 15uM in 0.1M sodium phosphate pH 7.0) was mixed with 0.0056ml of Solution A to give a theoretical MSWP-SH molar excess of five-fold over protein. The mixture was left for 4h at room temperature followed by 18h at 4 degrees C. Analysis by SDS PAGE followed by protein staining indicated one major band at apparent M_r 23K, corresponding to unreacted protein, and a minor band at apparent M_r 26K, corresponding to title protein.

Method (b)

TNB-activated SCR1-3/switch product (SEQ ID NO: 7) (10uM; 0.43ml) prepared in a similar way to that described in Example 7(d) was mixed with TCEP (5mM; 0.0026ml) and incubated for 17h at room temperature to yield the free fusion protein SEQ ID NO: 7. MSWP-1 (10mM; 0.0086ml) was added and incubation was continued for a further 4h. Small particles or crystals were present in the solution, but it was otherwise clear. The particulate solution was buffer-exchanged into 50mM formic acid (1.0ml), aliquoted and frozen. Analysis by SDS PAGE under non-reducing conditions showed a number of bands, which included a species with an apparent molecular weight of 25 000 - the target species.

Example 11 Preparation of [CR1: 1-1929]-Cys-S-S-[MSWP-1] (SEQ ID NO: 10)

H_2N-[CR1(1-1929)]-Cys-OH
|
S
|
S
|
N-(myristoyl)-Gly-Ser-Ser-Lys-Ser-Pro-Ser-Lys-Lys-Lys-Lys-Lys-Lys-Pro-Gly-Asp-Cys-NH_2

Human complement receptor 1 (CR1, CD35) is a known regulator of complement activation which has been produced in a recombinant soluble form containing all of the extracellular SCR domains of a major natural allotype (Fearon et al, WO 89/09220, WO 91/05047). This form (sCR1) has been expressed as an active protein in Chinese Hamster Ovary (CHO) cells. Mutagenesis of the DNA sequence immediately downstream of the codon for Cys-1924 is performed to generate a new C-terminal cysteine residue.

A suitable example of a modified terminus of the cDNA sequence of sCR1 is as follows:

(5909) Bal I (5914)
.....CCT CTG GCC AAA TGT ACC TCT CGT GCA CAT TGC TGA

The codon Asp-1930 in CR1 is replaced by that for a Cysteine (followed by a stop codon to generate a soluble protein) through ligation of a modified oligonucleotide to the unique Bal I restriction endonuclease site at position 5914 (numbering from Fearon et al, 1989,1991).

Expression of this modified cDNA in CHO cells and isolation of the product by standard chromatographic procedures generates a modified sCR1 protein which can be treated as in Example 8(a), (b) or (c) to couple it to MSWP-1 (Example 2) to yield the title compound.

Example 12 Preparation of [SCR1-3]-Cys-S-S-[MSWP-2] (SEQ ID No. 11)

H_2N-[SCR 1-3]-Cys-OH
|
S
|
S
|
N-acetyl-Cys-Asp-Gly-Pro-Lys-Lys-Lys-Lys-Lys-Lys-Ser-Pro-Ser Lys-Ser-Ser-Lys-(N-Myristoyl)-NH_2

[SCR1-3]-Cys protein (SEQ ID NO: 6) prepared in a similar way to that described in Example 6(d) (46uM protein; 0.20ml) was mixed with TCEP (5mM;

0.0054ml) and incubated at room temperature for approx. 20h. 0.05ml of this solution was mixed with 0.025ml of 0.1M ethanolamine and 0.003ml of MSWP-2 (see Example 3; 5mM in DMSO;); the solution was incubated for a further 3h at room temperature. SDS PAGE analysis showed the major band in the preparation had an apparent molecular weight of 23 000, clearly shifted from the original parent molecular weight of 21 000. The purity of the target protein was estimated from the SDS PAGE gel to be approx 80%.

Example 13 Preparation of [SCR1-3]-Cys-S-S-[MSWP-3] (SEQ ID No. 12)

H_2N-[SCR 1-3]-Cys-OH
|
S
|
S
|

N-(Myristoyl) -Ser-Lys-Asp-Gly-Lys-Lys-Lys-Lys-Lys-Lys-Ser-Lys-Thr-Lys-Cys-NH_2

[SCR1-3]-Cys protein (SEQ ID NO: 6) prepared in a similar way to that described in Example 6(d) (46uM protein; 0.10ml) was mixed with TCEP (5mM; 0.0037ml) and incubated at room temperature for approx. 18h. 0.01ml of 0.5M ethanolamine was added. 0.03ml of this 0.11ml solution was mixed with 0.0032ml of MSWP-3 (see Example 4; 2mM in 0.1M sodium phosphate pH 7.0); the solution was incubated for a further 3h at room temperature. SDS PAGE analysis showed the major band in the preparation had an apparent molecular weight of 23 000, clearly shifted from the original parent molecular weight of 21 000. The purity of the target protein was estimated from the SDS PAGE gel to be approx 80%.

Example 14 Preparation of [SCR1-3]-Cys-S-S-[TCPT-1] (SEQ ID No. 13)

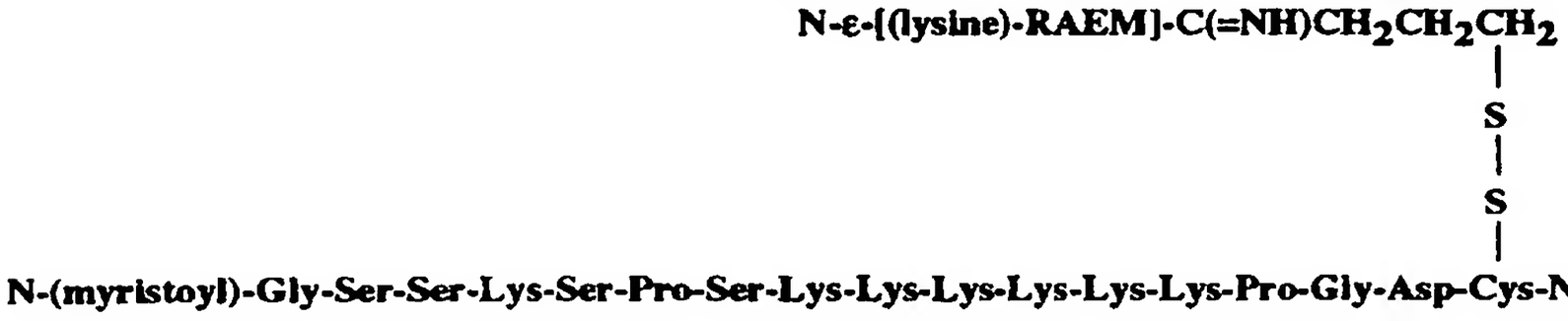
H_2N-[SCR 1-3]-Cys-OH
|
S
|
S
|

N-acetyl-Cys-Ser-Ala-Ala-Pro-Ser-Ser-Gly-Phe-Arg-Ile-Leu-Leu-Leu-Lys-Val-$NH(CH_2)_9CH_3$

[SCR1-3]-Cys protein prepared in a similar way to that described in Example 6(d) (46uM protein; 0.08ml) was mixed with TCEP (5mM; 0.0029ml) and incubated at room temperature for approx. 18h. 0.008ml of 0.5M ethanolamine was added. 0.04ml of this 0.088ml solution was mixed with 0.0029ml of TCPT-1 (see Example 5; 2.9mM in

DMSO). The TCPT-1 was added in 6 aliquots over a 2h period to minimise aggregation. The solution was incubated for a further 2h at room temperature. The final appearance of the mixture was one of a colloidal suspension and centrifugation at 2000g for 1 min showed that the target protein was compartmentalised in the precipitate. SDS PAGE analysis showed the major band in the preparation had an apparent molecular weight of about 23 000, clearly shifted from the original parent molecular weight of 21 000. The purity of the target protein was estimated from the SDS PAGE gel to be approx 80%.

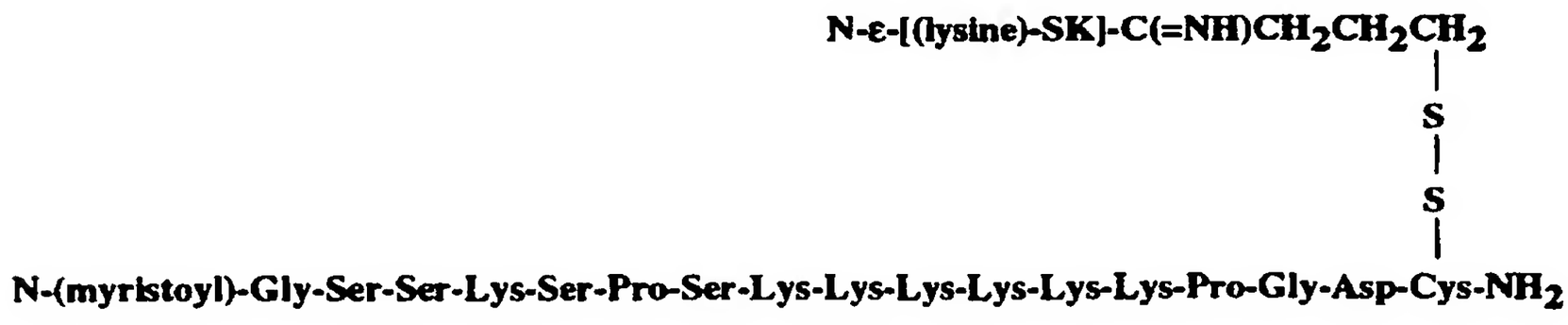
Example 15 Preparation of a Rabbit anti-(human erythrocyte membrane) antibody - [MSWP-1] conjugate (RAEM-MSWP-1) (SEQ ID NO: 32)



Rabbit polyclonal anti(human erythrocyte membrane) (RAEM) antiserum (Dako, Denmark, 13mg/ml, 0.25ml) was diluted to 1.0ml with 50mM sodium phosphate 0.1M sodium chloride pH 7.4 (PBS) and treated with 30 ul of 100mM 2-iminothiolane in PBS (freshly dissolved) for 30 min at 25°C. These conditions have been shown (R.A.G.Smith & R.Cassels, Fibrinolysis, 2,189-195, 1988) to introduce an average of 2-3 free thiol groups per molecule of immunoglobulin G.

The product was purified by gel permeation chromatography on a small disposable column of Sephadex G-25m (PD-10, Pharmacia, Stockholm,Sweden) at 4°C. 2.5ml of the product (total volume 3.0ml, theoretical protein concentration ~6.1 uM) was treated with MSWP-1 (Example 2, 0.125 ml of 5mM solution in dimethyl sulphoxide, final conc ~240uM) and incubated at 25°C for 30 min. The product was gel-filtered on a PD10 column as above to give 3.0ml of a solution ~5uM in protein. This was stored frozen at -70°C.

Example 16 Preparation of a conjugate of Streptokinase and MSWP-1 (SEQ ID No 21)



Streptokinase (SK) stock solution (Behringwerke, Marburg, Germany, 12.8 mg/ml, 271 uM, 2.5ml) was gel filtered using a PD10 column into 3.2 ml of PBS buffer (see Example 15) containing 0.01%w/v Tween 80 [PST buffer]. Freshly made up 2-iminothiolane (64ul of 100mM) was added and the mixture incubated at 25°C for 1h. The product was gel filtered in 2 x 1.6ml batches into 2x 3.0 ml PST at 4°C on two PD10 columns. This solution was stored in aliquots of 1.5ml at -75°C.

Titration of the product with Ellman's reagent (0.1mM in 0.5ml 0.1M Triethanolamine.HCl pH 8.0) showed that it contained approximately 0.3mM free thiol groups. This corresponds to an average of 3- 3.5 thiol groups per molecule of SK. The stock thiolated SK solution (2 x 0.5ml) was processed by modifying one aliquot with MSWP-1 (32ul of 5mM stock in DMSO), incubated 1h at 25°C and gel filtered (PD10 column) into 3.0ml PST at 4°C. A control aliquot was processed in parallel without exposure to MSWP-1. Both products contained ~ 0.8 mg/ml protein based on an extinction coefficient of 0.76 $(mg/ml)^{-1}$ at 280nm for SK and were stored at -75°C.

Example 17 Reversible linkage of MSWP-1 to the active centre of Human Tissue-type Plasminogen Activator (SEQ ID No 22)

tPA [Ser-478]-O-4-benzoyl-$NH(CH_2)_2NHCO(CH_2)_2$-S

|

S

|

N-(myristoyl)-Gly-Ser-Ser-Lys-Ser-Pro-Ser-Lys-Lys-Lys-Lys-Lys-Lys-Pro-Gly-Asp-Cys-NH_2

The thiol-reactive acyl-enzyme 4-N-[2-N-(3-[2-pyridyldithio]-ethylcarbonyl)aminoethyl]aminobenzoyl - [Ser-478] human tissue-type plasminogen activator [PDAEB->tPA] was prepared by the method of Smith and Cassels (Fibrinolysis, 2, 189-195, 1988). Tissue plasminogen activator (Actilyse, Boehringer Ingelheim,Germany, approx 2mg) was dissolved in the PST buffer of Example 16 (1.0ml) and treated with 25ul of a 20mM solution of 4'-amidinophenyl 4-N-[2-N-(3-[2-pyridyldithio]-ethylcarbonyl)aminoethyl]aminobenzoate hydrochloride (S.B.Kalindjian & R.A.G.Smith, Biochem. J. 248, 409-413,1987) in dimethylsulphoxide. The mixture was incubated for 1h at 25°C and stored frozen at -80°C. It was reduced by addition of dithiothreitol (5ul of 0.5M in water) for 30 min at 0°C followed by buffer-exchange into PST buffer (3.0ml) as described in Example 16. The product was divided immediately into a retained sample (0.6ml) and a reaction sample (2.4ml) which was mixed with MSWP-1 (Example 2, 100ul of a 5mM solution in dimethylsulphoxide) and incubated for 90 min on ice. The product was buffer-exchanged as above into 3.2ml PST and stored in aliquots at -196°C.

Example 18 Expression and purification of [SCR1-3]-Cys (SEQ ID 6) from a fermentation run.

(a) Fermentation of *E. coli* harbouring the plasmid pDB1030

A frozen stock of *E. coli* harbouring the plasmid pDB1030 was initially prepared by plating the culture out onto LB agar plus ampicillin at 100µg/ml. 1ml aliquots were preserved in a 10% glycerol / PBS cryopreservative and stored under liquid nitrogen. A 1ml vial was thawed and was used to inoculate 100ml LB^{Amp100} primary seed medium (Difco Bactotryptone, 10gl^{-1}; Difco yeast extract, 5gl^{-1}; sodium chloride, 5gl^{-1}; pH pre-sterilisation 7.4) in a 500ml flask. The primary seed stage was incubated at 37°C for 3 hours before transfer to the second seed stage, also 100ml LB^{Amp100} per 500ml flask using a 1% inoculum. Following incubation as above for a further 4 hours a 1% inoculum was transferred to the tertiary seed stage, 10litres LB^{Amp100} in a 15litre Biolafitte fermenter. The 10 litres tertiary seed medium was sterilised *insitu* for 45 minutes at 121°C before inoculation. Following incubation for 14.5 hours, the tertiary seed was transferred to the final stage fermenter as a 6% inoculum. Incubation conditions for the seed stage were as follows: airflow at 10lmin^{-1} (1.0vvm), temperature 37°C, agitation at 400rpm (1.9ms^{-1}) and overpressure 0.2bar. 300 litres Tryptone phosphate $medium^{Amp100}$(Difco Bactotryptone, 20gl^{-1}; Difco yeast extract, 15gl^{-1}; sodium chloride, 8gl^{-1}; disodium hydrogen orthophosphate, 2gl^{-1}; potassium dihydrogen orthophosphate, 1gl^{-1}; Dow Corning 1520 antifoam, 0.1gl^{-1}; pH pre-sterilisation 7.4) was sterilised *in situ* for 30 minutes at 121°C in a 450L Bioengineering fermenter. The fermenter was inoculated with 20 litre inoculum from the tertiary seed stage and incubated under the following conditions : airflow 450L min^{-1} (1.5vvm), temperature 37°C, agitation 230rpm (1.5ms^{-1}) and overpressure 0.5bar. After an OD_{550nm} of 3.5 was obtained, 1mM IPTG was added. Harvest followed after continued incubation for 2 hours. A cell slurry was recovered after primary centrifugation through a Westfalia CSA19 (two discharges). The cells were further spun at 4700rpm (7000g) for 30 minutes in a Sorvall RC3B centrifuge. The total cell yield (wet weight) was 2.98Kg and was stored at -80 degrees C in approx. 600g lots.

(b) Isolation of inclusion bodies and purification of [SCR1-3]-Cys

Inclusion bodies from 100 g (wet weight) cell pellet were isolated and solubilised essentially as described in Example 6. The purification of target protein from resolubilised inclusion bodies was also as described in Example 6 with some modifications. The major ones were :

1. The use of Macroprep High S (Biorad) instead of S-Sepharose. 200g of matrix was used for 100g of cell pellet that had been sonicated. 1.4g of approx. 60%

pure target protein was produced in the solubilised and partially purified fractionon bodies.

2. Refolding of a 100mg sample of the partially purified protein was carried out by diluting the fully denatured protein (2mg/ml) 100-fold in cold 60mM ethanolamine/1mM EDTA, followed by addition of the glutathione redox couple.

The product of the above process was capable of being modified with MSWP-1 (Example 2) in a way similar to that described in Example 8.

Example 19 Expression and isolation of [SCR1-3(delN195-K196)]TNANKSLSSISCQT (SEQ ID NO: 14)

(a) Construction of plasmid pBC04-29 encoding [SCR1-3(delN195-K196)]TNANKSLSSISCQT

Plasmid pBC04-29 was constructed from plasmid pDB1013-5 encoding SCR1-3 of LHR-A of CR1 (patent application WO 94/00571) by QuickChange site directed mutagenesis (Stratagene) according to the manufacturers protocols. Two complementary oligonucleotides (SEQ ID No 15 and SEQ ID No 16 were used to generate a novel restriction site (silent) at G186/P187 and a C terminal cysteine. In the event the reaction produced a frame-shift mutation at position N195. In the resulting sequence the C terminal amino acids N195 and K196 are replaced with a 14 amino acid peptide TNANKSLSSISCQT. Fortuitously, this sequence contains an internal cysteine close to the C terminus, preceeded by a spacer sequence of 11 amino acids.

(b) Expression of plasmid pBC04-29 encoding [SCR1-3(delN195-K196)]TNANKSLSSISCQT in *E. coli*

pBC04-29 was transformed into competent *E. coli* BL21(DE3)pLys-S and resultant colonies were isolated and checked for plasmid content. A single colony was inoculated into 10 ml LB medium (10g/L bactotryptone, 5g/L yeast extract, 10g/L NaCl) containing 50ug/ml ampicillin. The culture was grown for 6 -18 hours at 37°C, 230 r.p.m. before being used to inoculate 1 litre of the same medium containing 50 ug/ml ampicillin at a dilution of 1 in 100 in 4 L erlenmeyer flasks. Cells were grown to an OD of 0.8 - 1.0 at A_{600} nm. IPTG (isopropyl B-D galactopyranoside) was added to a final concentration of 1 mM and cells allowed to continue growth for a further 3 - 4 hours or overnight before harvesting by centrifugation at 8000 g/10 min. Pellet from 1L of culture was stored at -80°C.

(c) Isolation and purification of [SCR1-3(delN195-K196)]TNANKSLSSISCQT

The methods are essentially those detailed in Dodd I. et al (1995) Protein Expression and Purification 6 727-736, subsequently modified as described in Example 18. Briefly, the cell pellet from 1L of culture from (b) was resuspended in buffer, sonicated and the inclusion bodies isolated by centrifugation. The inclusion bodies were resolubilised in 100 ml of fully reducing buffer and target protein purified on Macroprep High S (30g wet weight). Product (27ml at nominal 1.5mg/ml) that eluted from the column in the 1M NaCl-containing buffer was refolded by dilution into 2.5L cold 60mM ethanolamine/1mM EDTA, with the glutathione redox agents added at 1h. After 3d at 4 degrees C the solution was ultrafiltered using a YM10 membrane and the retentate was treated with ammonium sulphate, centrifuged and the supernatant purified on Butyl Toyopearl 650M (bed volume 53ml). A single A280 peak was eluted by the decreasing ammonium sulphate gradient. SDS PAGE under non-reducing conditions followed by protein staining revealed a major polypeptide with an apparent molecular weight of 22 000, believed to be the target protein. One of the contaminating polypeptides had an apparent molecular weight of about 40 000, which was identified as the dimer of the monomeric form of the target by comparison with adjacent markers of [SCR1-3]-Cys. The product had an estimated protein concentration of 30uM.

Example 20 Preparation of [SCR1-3(delN195-K196)]TNANKSLSSISC-(-S-S-[MSWP-1])QT (SEQ ID No. 17)

H_2N-[SCR 1-3(delN195-K196)]-Thr-Asn-Ala Asn- Lys-Ser-Leu-Ser-Ser-Ile-Ser-Cys-Gln-Thr

|

S

|

S

|

N-(myristoyl)-Gly-Ser-Ser-Lys-Ser-Pro-Ser-Lys-Lys-Lys-Lys-Lys-Lys-Pro-Gly-Asp-Cys-NH_2

[SCR1-3(delN195-K196)]TNANKSLSSISCQT prepared as described in Example 19 (approx. 30uM protein; 0.1ml) was mixed with TCEP (5mM in 50mM Hepes pH 4.5; 0.0072ml) and incubated at room temperature (22 degrees C) for 15h. 0.05ml of this solution was mixed with 0.005ml of 0.5M ethanolamine and 0.003ml of 7mM MSWP-1 (see Example 2); the solution was incubated for a further 4h at room temperature. SDS PAGE analysis showed a major band in the preparation had an apparent molecular weight of 25 000, clearly shifted from the original parent molecular weight of 23 000.

Example 21 Preparation of [SCR1-3]DGPSEILRGDFSSC (SEQ ID No. 23)

(a) Construction of plasmid pBC04-31 encoding [SCR1-3]DGPSEILRGDFSSC

Plasmid pBC04-31 was constructed using plasmid pBC04-29 (described in Example 19) and a synthetic oligonucleotide pair (SEQ ID No. 25 and SEQ ID No. 26). pBC04-29 was digested with the restriction enzymes HindIII and ApaI and the large fragment (2170bp) isolated. The two oligonucleotides were annealed by warming to >90oC and slowly cooling to room temperature and ligated with the DNA fragment. The ligated DNA was transformed into competent *E. coli* XLI-Blue. Colonies were analysed for plasmids in which the oligonucleotides had been inserted by looking for the presence of a novel AvaI site at position 2733. On digestion with AvaI pBC04-31 yielded fragments of 2311 and 495bp. DNA from positive clones was used to transform the expression strains. The oligonucleotides inserted added the peptide sequence DGPSEILRGDFSSC to the C terminus of SCR1-3 and also repaired the frame-shift error seen in pBC04-29.

(b) Expression, isolation and purification of [SCR1-3]DGPSEILRGDFSSC

Expression, isolation and purification of **[SCR1-3]DGPSEILRGDFSSC** is carried out using pBC04-31 by procedures generally described in Example 6.

Example 22 Preparation of [SCR1-3]DGPSEILRGDFSSC-(-S-S-[MSWP-1]) (SEQ ID No. 24)

H_2N-[SCR 1-3]-Asp-Gly-Pro-Ser-Glu-Ile-Leu-Arg-Gly Asp-Phe-Ser-Ser-Cys-OH
|
S
|
S
|
N-(myristoyl)-Gly-Ser-Ser-Lys-Ser-Pro-Ser-Lys-Lys-Lys-Lys-Lys-Lys-Pro-Gly-Asp-Cys-NH_2

[SCR1-3] DGPSEILRGDFSSC protein prepared in a similar way to that described in Example 21 is reacted with MSWP-1 as described in Example 8 to give the title compound.

Example 23 Preparation of [SCR1-3] AAPSVIGFRILLLKVAGC (SEQ ID No. 33)

(a) Construction of plasmid pBC04-34 encoding [SCR1-3] AAPSVIGFRILLLKVAGC

Plasmid pBC04-34 was constructed using plasmid pBC04-29 (described in Example 19) and a synthetic oligonucleotide pair (SEQ ID No. 34 and SEQ ID No. 35). pBC04-29 was digested with the restriction enzymes HindIII and ApaI and the large fragment (2170bp) isolated. The two oligonucleotides were annealed by warming to >90°C and slowly cooling to room temperature and were ligated with the DNA fragment. The ligated DNA was transformed into competent *E. coli* XLI-Blue. Colonies were analysed for plasmids in which the oligonucleotides had been inserted by looking for an increase in size of the Nde1/HindIII fragment by 59 base pairs. The presence of the cysteine codon was determined by the presence of a DdeI site at position 2781. pBC04-34 digested with DdeI yielded diagnostic bands of 481 and 109bp DNA from positive clones was used to transform the expression strains (see next section). The oligonucleotides inserted added the peptide sequence AAPSVIGFRILLLKVAGC to the C terminus of SCR1-3 and also repaired the frame-shift error seen in pBC04-29.

(b) Expression,isolation and purfication of [SCR1-3]APSVIGFRILLLKVAGC

Expression isolation and purification of [SCR1-3] APSVIGFRILLLKVAGC is carried out using pBC04-34 by procedures generally described in Example 6.

Example 24 Preparation of [SCR1-3]AAPSVIGFRILLLKVAGC -(-S-S-[MSWP-1]) (SEQ ID No. 36)

H_2N-[SCR 1-3]-Ala-Ala-Pro-Ser-Val-Ile-Gly-Phe-Arg-Ile-Leu-Leu-Leu-Lys-Val-Arg-Gly-Cys-OH
|
S
|
S
|
N-(myristoyl)-Gly-Ser-Ser-Lys-Ser-Pro-Ser-Lys-Lys-Lys-Lys-Lys-Lys-Pro-Gly-Asp-Cys-NH_2

[SCR1-3]AAPSVIGFRILLLKVAGC protein prepared in a similar way to that described in Example 23 is reacted with MSWP-1 as described in Example 8.

Biological Activity

(I) Anti-complement Activity Measured by the Classical Pathway-mediated Haemolysis of Sheep Erythrocytes

(i) Functional activity of complement inhibitors was assessed by measuring the inhibition of complement-mediated lysis of sheep erythrocytes sensitised with rabbit antibodies (Diamedix Corporation, Miami, USA). The assay is designed to be specific for the classical pathway of complement activation. Human serum diluted 1:500 or 1:400 (final concentration in assay mixture) in 0.1 M Hepes/0.15 M NaCl/0.1% gelatin pH 7.4 was used as a source of complement. The serum was prepared from a pool of volunteers essentially as described in Dacie & Lewis, 1975. Briefly, blood was warmed to 37°C for 5 minutes, the clot removed and the remaining serum clarified by centrifugation. The serum fraction was split into small aliquots and stored at -196°C or -80°C. Aliquots were thawed as required and diluted in the Hepes buffer immediately before use.

Inhibition of complement-mediated lysis of sensitised sheep erythrocytes was measured using a standard haemolytic assay using a v-bottom microtitre plate format as follows:

50 µl of a range of concentrations of inhibitor diluted in Hepes buffer were mixed with 50 µl of the diluted serum and 100 µl of sensitised sheep erythrocytes and then incubated for 1 hour at 37°C. Samples were spun at 1600rpm at ambient temperature for 3 minutes before transferring 150 µl of supernatant to a flat bottom microtitre plate and determining the absorption at 405 or 410 nm. Maximum lysis (Amax) was determined by incubating serum with erythrocytes in the absence of any inhibitor. Background lysis (Ao) was determined by incubating erythrocytes in the absence of any serum or inhibitor. Inhibition was expressed as a fraction of the total cell lysis such that IH50 represents the concentration of inhibitor required to give 50% inhibition of lysis.

$$\% \text{ inhibition} = 1 - [(A - Ao)/(Amax - Ao)]$$

Results

Compound	IH50
WO94/00571 SEQ ID NO 27	0.2 - 0.3 ug/ml [10-15 nM] (1)
Example 6*	0.65 ug/ml [30nM] (mean of two) (2)
Example 7*	0.3-1.0 ug/ml [15-50nM] (n = 3)
Example 8a Example 8b Example 8c Example 8d+	0.014 ug/ml [0.6 nM]) <0.001 ug/ml [< 0.04nM]) (3) 0.001 ug/ml [0.043 nM]) [0.06nM])
Example 10a Example 10b Example 12 Example 13 Example 14	0.02 ug/ml [0.8nM] ~0.01 ug/ml [~0.4nM] ~0.0016 ug/ml [0.07 nM] ~0.009 ug/ml [0.4nM] ~1.1 ug/ml [50nM]
Example 19	[4nM]

* As 2-mercaptoethanol/glutathione derivatives

+ Assay of the two solutions and the original pre-lyophilisation solution from Example 8d.

Other IH_{50} values generated for similar batches include:

(1) 15nM

(2) 8nM, 5nM; 8nM, 4nM

(3) 0.3nM, 0.2nM, 0.07nM, 0.06nM, 0.2nM, 0.4nM, 0.5nM, 0.6nM.

The above data show that:

1. The complement inhibitory activities of the 'base' protein (SCR1-3 of human complement receptor 1 of WO94/00571) and its derivatives with either an additional C-terminal cysteine (SCR1-3/cys, Example 6) or a single cationic 'switch' sequence (SCR1-3/switch, Example 7) are similar.

2. However, incorporation of two membrane binding elements (electrostatic switch and myristoyl) by addition of MSWPs-1, 2 or 3 (which contain both elements) to SCR1-3/cys or three membrane binding elements by addition of the MSWP-1 to the SCR1-3/switch construct results in products which are significantly more potent (~20-200x) than the base or single membrane binding element proteins. The use of TCTP-1 which is targeted to membrane elements found in CD3-positive cells and not to erythrocyte membranes gave a conjugate of similar potency to SCR1-3 derivatives with no or single

membrane addresses. Thus, the increases in potency in an assay which depends on an erythrocyte membrane event (cytolysis by the membrane attack complex of complement) can be attributed to membrane targeting of the cytolysis inhibitor proteins by the combination of two membrane binding elements.

(ii) Assay of anti-complement activity in the classical pathway haemolytic assay: activity in the sera of domestic pig, guinea pig, rat and marmoset.

The activity of [SCR1-3]-Cys-S-S-[MSWP-1] was examined in the classical pathway haemolytic assay using the sera of pig, guinea pig, rat or marmoset. The methodology was essentially as described in (I) with minor modifications, for example small changes to the concentration of serum used. [SCR1-3]-Cys-S-S-[MSWP-1] was prepared essentially as described in Example 8c. The IH50 values for the different sera were: pig, 0.2nM; guinea pig, 0.3nM; rat, 0.4nM; marmoset, 0.2nM. These results show that [SCR1-3]-Cys-S-S-[MSWP-1] is capable of inhibiting classical pathway complement inhibition in the sera of a variety of animal species.

(II) Anti-complement Activity Measured by Alternative Pathway-mediated Haemolysis of Guinea Pig Erythrocytes

Functional activity of complement inhibitors was assessed by measuring the inhibition of complement mediated lysis of guinea pig erythrocytes essentially as described by Scesney, S. M. et al (1996) J. Immunol. 26 1729-1735. The assay is designed to be specific for the alternative pathway of complement activation. Human serum prepared from a pool of volunteers essentially as described in Dacie & Lewis, 1975 was used as the source of complement. Briefly, blood was warmed to 37°C for 5 minutes, the clot removed and the remaining serum clarified by centrifugation. The serum fraction was split into small aliquots and stored at -196°C or -80°C. Aliquots were thawed as required and diluted in 0.1 M Hepes/ 0.15 M NaCl / 0.1% gelatin / 8 mM EGTA / 5 mM $MgCl_2$ pH 7.4 (buffer A) immediately before use. Guinea pig erythrocytes were prepared from guinea pig whole blood collected into EDTA-coated tubes as follows. The blood was spun at 1600 rpm for 5 min and the erythrocyte pellet washed 3 times with 0.1 M Hepes/ 0.15 M NaCl / 0.1% gelatin pH 7.4 until the supernatant of the spin was essentially colourless. The erythrocytes were finally resuspended to the original volume of blood used and were stored at + 4 degrees C. They were used within 2 weeks.

50 µl of a range of concentrations of inhibitor diluted in buffer A in a v-bottom microtitre plate were mixed with, first,100 µl of serum that had been diluted 1:3 and second, 50 µl of guinea pig erythrocytes (diluted 1:49 in buffer A) and incubated for 1

hour at 37°C. The plate was spun at 1600 rpm for 3 minutes before transferring 150 µl of each supernatant to a flat bottom microtitre plate and determining the absorption at 405 nm, which reflects the amount of lysis in each test solution. Maximum lysis (Amax) was determined by incubating serum with erythrocytes in the absence of any inhibitor. Background lysis (Ao) was determined by incubating erythrocytes in the absence of any serum or inhibitor. The final dilution of serum used in the assay did absorb at 405nm but the level of absorbance (approx 10% of Amax) was considered to have a neglible affect on the overall assay results and it was ignored in the calculations. Inhibition was expressed as a fraction of the total cell lysis such that IH50 represents the concentration of inhibitor required to give 50% inhibition of lysis.

$$\% \text{ inhibition} = 1 - [(A - Ao) / (Amax - Ao)]$$

Results

Two aliquots (one lyophilised and resolubilised in a neutral buffer, the other not lyophilised) of a single batch of of [SCR1-3]-Cys-S-S-[MSWP-1] prepared in a similar way to that described in Example 8 (c) were tested in the haemolytic assay. The IH50 values for the compounds were:

[SCR1-3]-Cys-S-S-[MSWP-1] (not lyoph)	310nM
[SCR1-3]-Cys-S-S-[MSWP-1] (lyoph)	480nM

The result shows that [SCR1-3]-Cys-S-S-[MSWP-1] exhibited activity against the alternative pathway of the complement system and that lyophilisation and subsequent resolubilisation of the protein had no affect (within experimental error) on the biological activity of the protein.

(III) Plasminogen activator assay

(i) SK-related molecules from Example 16 were assayed using a plasminogen activation assay. A solution of purified human Lys_{77}-Plasminogen (1uM in PST buffer containing 25%v/v glycerol [PSTG buffer], 0.5ml) was incubated with thiolated SK (final concentration 0.1 to 1.07 nM) for 1h at 25°C. An aliquot of this mixture (10ul) was incubated with 1.0mM of the plasmin substrate S-2251 (H-D-Val-Leu-Lys-p-nitroanilide, KabiVitrum, Stockholm, Sweden) in 0.1M Triethanolamine HCl pH 8.0 (0.5ml) at 25°C. The release of p-nitroaniline was monitored continously at 405nm. Under these conditions, one substrate unit (SU) of plasmin activity is defined as the amount of enzyme giving an increase in optical density at 405nm of 0.001 min^{-1}. Under these conditions thiolated SK (1nM) generated plasmin at a nearly linear of 4225 SU/ml.

SK-MSWP-1 conjugate was diluted 1:100 in PSTG buffer and 5-50ul aliquots tested in the plasminogen activation assay. The stock preparation was found to contain approximately 2.9uM functional SK.

(ii) The potential activity of the acyl-enzyme preparations of Example 17 was estimated by dilution 25-50 fold into PST buffer and incubation for 2h at 37°C, followed by assay using 2mM S-2288 (H-D-Ile-Pro-Arg-p-nitroanilide 2HCl) under the same conditions used in (i) above. Under these conditions, the potential activity of the reduced PDAEB->tPA preparation was 2760 SU/ml and that of the MSWP-1/PDAEB->tPA conjugate 535 SU/ml.

(IV) Erythrocyte binding assays

(i) Erythrocyte aggregation test for modified and unmodifed Rabbit anti-(human erythrocyte membrane) antibody.

Human pooled erythrocytes (Ortho A2, Raritan, New Jersey, 3% v/v, 50ul) were added to microtitre plates wells and either unmodified rabbit anti-(human erythrocyte membrane) antibody [RAEM] or RAEM-MSWP1 conjugate from Example 15 added at concentrations expressed relative to undiluted stock RAEM. Cells were agitated at ~100rpm for 40min at 25°C. 5ul was removed from each well and examined by light microscopy at x 20 magnification. A visual scoring scale was used as follows:

- \- No clumping, cells moving freely relative to each other.
- \+ Small clumps (<10 cells)
- ++ Larger clumps (100 plus cells)
- +++Very large visible aggregates

<u>Results</u>

Controls (n=6)	-	RAEM-MSWP1 1/3900	+/-
RAEM 1/1100	-	RAEM-MSWP1 1/1000	+/-
RAEM 1/600	-		
RAEM 1/350	+/-	RAEM-MSWP1 1/357	+++
RAEM 1/50	++	RAEM-MSWP1 1/62	+++

<u>Conclusion</u>

The antibody preparation modified to contain a membrane-binding unit was more effective at inducing aggregation of cells because binding to the cell membrane through MSWP1 allowed a higher effective concentration of bridging antibody on the membrane surface.

(ii) Binding of 125-Iodine-[SCR1-3]-Cys-S-S-[MSWP-1] to human erythrocytes

[SCR1-3]-Cys-S-S-[MSWP-1] (2mg/ml in PBS; 0.25ml) was mixed with 0.5mCi of 125-Iodine (Amersham) in the presence of 9nmoles potassium iodide following the Iodogen procedure and reagents (Pierce and Warriner (UK) Ltd.). The labelling was allowed to proceed for 20 min at room temperature, the reaction was quenched with 0.1ml of 1M potassium iodide and the solution buffer-exchanged into PBS/0.1% albumin. Citrated blood collected from a healthy volunteer was used as a source of human erythrocytes. Blood (0.2ml) was mixed with 10 microlitres of appropriately diluted 125-Iodine-[SCR1-3]-Cys-S-S-[MSWP-1] (final concentration 700pM) and incubated for 30min at 37 degrees C. The erythrocytes were then isolated by three repeat washings in PBS / centrifugation steps and samples counted in a Wallac 1470 Wizard gamma counter. The results were as follows:

	cpm
1st wash`	3 600 000
1st pellet	140 000
2nd wash	52 000
3rd wash	6 500
final pellet	26 000

Using values of 5 X 10^9 erythrocytes per ml of blood and and a specific radioactivity of 2.7 X 10^7 cpm / nmole for the [SCR1-3]-Cys-S-S-[MSWP-1] it was calculated that about 600 molecules of [SCR1-3]-Cys-S-S-[MSWP-1] bound per cell (the value for 'final pellet').

(iii) Binding of fluorescein-labelled-[SCR1-3]-Cys-S-S-[MSWP-1] to human erythrocytes

[SCR1-3]-cys (prepared in a similar way to that described in Example 18) (45uM,1.0mg/ml in 0.1M sodium phosphate, approx. 0.2M ammonium sulphate pH 7.0) was partially reduced by incubation at 25°C for 18h by the addition of a 4-molar excess of Tris(2-carboxyethyl)phosphine (TCEP; Pierce & Warriner (UK) Ltd.). The solution was buffer exchanged into 50mM Hepes pH7.0; post buffer exchange the protein concentration was 22uM. The partially reduced [SCR1-3]-cys was incubated with a 4-fold molar excess of 6-(fluorescein-5-carboxamido)hexanoic acid, succinimidyl ester (Molecular Probes Inc., USA) and incubated for 1h at 4°C. The excess fluorescent label was removed by buffer exchange of the protein solution into 50mM Hepes pH7.0. Fluorescein-[SCR1-3]-cys-S-S-[MSWP-1] was synthesised by adding MSWP-1 (Example 2) to give a five-fold molar excess over fluorescein labelled protein and was

incubated for 4h at 25°C. The solution was buffered exchanged into PBS and this solution was used for the microscopy studies.

[SCR1-3],10mg/ml in 50mM formic acid, was mixed in a 1:10 ratio with 50mM $NaHCO_3$ pH8.5; the pH of the solution was adjusted with NaOH to pH9.5. The fluorescein was extracted from Celite-fluorescein isothiocyante (Celite:fluorescein; 1:10, Sigma) by DMSO in a 1:4 (w/v) ratio. The fluorescein-DMSO solution was added to the protein solution in a 1:14 ratio and incubated for 1h at RT. Excess label was removed by gel filteration into PBS containing 0.01% Tween-80 and this solution was used for microscopy studies.

Citrated blood was collected from a healthy volunteer and the erythrocytes isolated, washed in PBS and diluted 250-fold compared to the original blood volume. 0.05ml of erythrocytes were incubated with 2uM fluorescein-[SCR1-3]-cys-S-S-[MSWP-1] or 2uM fluorescein-[SCR1-3] and incubated for 30min at 37°C. An eight microlitre sample of each incubation was mounted on a slide and viewed on an inverted confocal microscope (Biorad). The cells incubated with fluorescein-[SCR1-3] showed no specific staining whereas with those incubated with fluorescein-[SCR1-3]-cys-S-S-[MSWP-1] staining appeared diffusely over the cell surface and also intensely stained patches were visible on the cell membrane. No labelling was seen intracellularly.

(iv) Binding of MSWP-1/PDAEB->tPA to human erythrocytes

Human trypsinized and glutaraldehyde-treated red blood cells (1.0ml of a 4% suspension) was pelleted by low-speed centrifugation and resuspended in a total volume of 0.5ml PST containing either no additions or approximately 270 SU of either reduced PDEAB->tPA or MSWP-1/ PDAEB->tPA conjugate of Example 17. The mixtures were incubated by gentle rolling for 5 min at 23°C and then the cells were pelleted by centrifugation followed by two washes with 1.0ml PST buffer. Finally, the cells were suspended in 0.5ml PST and incubated at 37°C . Samples of the supernatant (100ul) were removed after pelleting. Assay using S-2288 (as above) showed that after 2h, approximately 7% of the applied t-PA activity was present in the supernatant of cells exposed to MSWP-1/PDAEB->tPA whereas only~2.8% was present in the supernatant of cells exposed to reduced PDAEB->tPA alone. No t-PA amidolytic activity was detected in controls.

This experiment suggests that reversible linkage of the active site of t-PA to MSWP-1 increases the tendency of this enzyme to bind to red blood cells.

(v) Localisation of SK-MSWP-1 conjugate on the surface of human erythrocytes

A stabilised preparation of human erythrocytes (trypsinised, glutaraldehyde-treated, Sigma, Gillingham,UK, 4% v/v, 0.4ml) was pelleted by centrifugation (~2000g/2min) and resuspended in 0.4ml PST buffer with either 0.1uM thiolated SK or 0.1uM SK-MSWP-1 from Example 16.

The suspensions were incubated for 30 min at 37°C and then washed by two cycles of centrifugation and resuspension in PST buffer. Finally, they were resuspended in PSTG buffer (0.4ml) containing 1uM plasminogen and incubated and assayed for plasmin as described above.

The control thiolated-SK generated plasmin at a rate of 522 SU/ml, while the SK-MSWP-1 conjugate produced 6184 SU/ml.The latter activity corresponds to around 2100 thiolated SK molecules/cell.

(vi) Binding of [SCR1-3]-Cys-S-S-[MSWP-1] to human erythrocyte membranes

4 X 2.0ml of trypsinized, glutaraldehyde-treated human erythrocytes (Sigma, R0127) were centrifuged for 2min at about 3000 rpm. The supernatants were discarded and the cells resuspended in phosphate/saline/Tween (0.01%) (PST) (1ml per tube) and [SCR1-3]-Cys-S-S-[MSWP-1] of Example 8 was added to a final concentration of 20ug/ml to three of the tubes. The mixtures were then incubated at 37 degrees C for 30 min., then washed five times by repeat centrifugation and washing in PST. The cells were finally suspended in 1ml PST and were held at 4 degrees C.

The ability of these cells to inhibit complement-mediated lysis of sheep erythrocytes was measured using the standard classical pathway complement inhibition assay described in (I) above. The human erythrocytes were added to the assay at four different dilutions, followed by the human serum and then the sheep red blood cells and incubation at 37 degrees C as usual. The % inhibition data are shown below.

Final dilution	human erythrocytes, untreated	human erythrocytes, treated with [SCR1-3]-Cys-S-S-[MSWP1]
1/4	22%	62%
1/16	-8%	88%
1/256	5%	74%
1/2500	-7%	51%

Thus the percentage inhibition for the [SCR1-3]-Cys-S-S-[MSWP-1]-treated cells at maximum dilution was significantly higher than the untreated cells at 1/4 dilution. The [SCR1-3]-Cys-S-S-[MSWP-1]-treated cells, therefore, contained at least 600-fold more complement inhibitory activity than the untreated cells, even though the cells had been washed extensively to remove any non-bound [SCR1-3]-Cys-S-S-[MSWP-1].

TABLE

SEQUENCE LISTING

(<- next to a peptide sequence in {} signifies sequence runs C to N terminus)

SEQ ID NO:1:

GCACCGCAGTGCATCATCCCGAACAAATGCTAATAAA

SEQ ID NO:2:

AGCTTTTATTAGCATTTGTTCGGGATGATGCACTGCG

SEQ ID NO:3:

GCACCGCAGTGCATCATCCCGAACAAAGACGGTCCGAAAAAGAAGAAAAAGAAATCTCCGTCCAAATCTTCC
GGTTGCTAATAAA

SEQ ID NO:4:

AGCTTTTATTAGCAACCGGAAGATTTGGACGGAGATTTCTTTTTCTTCTTTTTCGGACCGTCTTTGTTCGGG
ATGATGCACTGCG

SEQ ID NO:5:

Gly-Ser-Ser-Lys-Ser-Pro-Ser-Lys-Lys-Lys-Lys-Lys-Lys-Pro-Gly-Asp-Cys-NH_2

SEQ ID NO:6:

Met Gln Cys Asn Ala Pro Glu Trp Leu Pro Phe Ala Arg Pro Thr Asn
Leu Thr Asp Glu Phe Glu Phe Pro Ile Gly Thr Tyr Leu Asn Tyr Glu
Cys Arg Pro Gly Tyr Ser Gly Arg Pro Phe Ser Ile Ile Cys Leu Lys
Asn Ser Val Trp Thr Gly Ala Lys Asp Arg Cys Arg Arg Lys Ser Cys
Arg Asn Pro Pro Asp Pro Val Asn Gly Met Val His Val Ile Lys Gly
Ile Gln Phe Gly Ser Gln Ile Lys Tyr Ser Cys Thr Lys Gly Tyr Arg
Leu Ile Gly Ser Ser Ser Ala Thr Cys Ile Ile Ser Gly Asp Thr Val
Ile Trp Asp Asn Glu Thr Pro Ile Cys Asp Arg Ile Pro Cys Gly Leu
Pro Pro Thr Ile Thr Asn Gly Asp Phe Ile Ser Thr Asn Arg Glu Asn
Phe His Tyr Gly Ser Val Val Thr Tyr Arg Cys Asn Pro Gly Ser Gly
Gly Arg Lys Val Phe Glu Leu Val Gly Glu Pro Ser Ile Tyr Cys Thr
Ser Asn Asp Asp Gln Val Gly Ile Trp Ser Gly Pro Ala Pro Gln Cys
Ile Ile Pro Asn Lys Cys

SEQ ID NO:7:

Met Gln Cys Asn Ala Pro Glu Trp Leu Pro Phe Ala Arg Pro Thr Asn
Leu Thr Asp Glu Phe Glu Phe Pro Ile Gly Thr Tyr Leu Asn Tyr Glu
Cys Arg Pro Gly Tyr Ser Gly Arg Pro Phe Ser Ile Ile Cys Leu Lys
Asn Ser Val Trp Thr Gly Ala Lys Asp Arg Cys Arg Arg Lys Ser Cys
Arg Asn Pro Pro Asp Pro Val Asn Gly Met Val His Val Ile Lys Gly
Ile Gln Phe Gly Ser Gln Ile Lys Tyr Ser Cys Thr Lys Gly Tyr Arg
Leu Ile Gly Ser Ser Ser Ala Thr Cys Ile Ile Ser Gly Asp Thr Val
Ile Trp Asp Asn Glu Thr Pro Ile Cys Asp Arg Ile Pro Cys Gly Leu
Pro Pro Thr Ile Thr Asn Gly Asp Phe Ile Ser Thr Asn Arg Glu Asn
Phe His Tyr Gly Ser Val Val Thr Tyr Arg Cys Asn Pro Gly Ser Gly
Gly Arg Lys Val Phe Glu Leu Val Gly Glu Pro Ser Ile Tyr Cys Thr
Ser Asn Asp Asp Gln Val Gly Ile Trp Ser Gly Pro Ala Pro Gln Cys
Ile Ile Pro Asn Lys Asp Gly Pro Lys Lys Lys Lys Lys Lys Ser Pro
Ser Lys Ser Ser Gly Cys

SEQ ID NO:8:

linear, 2 polypeptide chains disulphide linked

Met Gln Cys Asn Ala Pro Glu Trp Leu Pro Phe Ala Arg Pro Thr Asn
Leu Thr Asp Glu Phe Glu Phe Pro Ile Gly Thr Tyr Leu Asn Tyr Glu
Cys Arg Pro Gly Tyr Ser Gly Arg Pro Phe Ser Ile Ile Cys Leu Lys
Asn Ser Val Trp Thr Gly Ala Lys Asp Arg Cys Arg Arg Lys Ser Cys
Arg Asn Pro Pro Asp Pro Val Asn Gly Met Val His Val Ile Lys Gly
Ile Gln Phe Gly Ser Gln Ile Lys Tyr Ser Cys Thr Lys Gly Tyr Arg
Leu Ile Gly Ser Ser Ser Ala Thr Cys Ile Ile Ser Gly Asp Thr Val
Ile Trp Asp Asn Glu Thr Pro Ile Cys Asp Arg Ile Pro Cys Gly Leu
Pro Pro Thr Ile Thr Asn Gly Asp Phe Ile Ser Thr Asn Arg Glu Asn
Phe His Tyr Gly Ser Val Val Thr Tyr Arg Cys Asn Pro Gly Ser Gly
Gly Arg Lys Val Phe Glu Leu Val Gly Glu Pro Ser Ile Tyr Cys Thr
Ser Asn Asp Asp Gln Val Gly Ile Trp Ser Gly Pro Ala Pro Gln Cys
-> <-
Ile Ile Pro Asn Lys Cys-S-S-{Cys Asp Gly Pro Lys Lys Lys Lys Lys
| |
CO_2H $CONH_2$
Lys Ser Pro Ser Lys Ser Ser Gly} (N-Myristoyl)

SEQ ID NO:9:

linear, 2 polypeptide chains disulphide linked

Met Gln Cys Asn Ala Pro Glu Trp Leu Pro Phe Ala Arg Pro Thr Asn
Leu Thr Asp Glu Phe Glu Phe Pro Ile Gly Thr Tyr Leu Asn Tyr Glu
Cys Arg Pro Gly Tyr Ser Gly Arg Pro Phe Ser Ile Ile Cys Leu Lys
Asn Ser Val Trp Thr Gly Ala Lys Asp Arg Cys Arg Arg Lys Ser Cys
Arg Asn Pro Pro Asp Pro Val Asn Gly Met Val His Val Ile Lys Gly
Ile Gln Phe Gly Ser Gln Ile Lys Tyr Ser Cys Thr Lys Gly Tyr Arg
Leu Ile Gly Ser Ser Ser Ala Thr Cys Ile Ile Ser Gly Asp Thr Val
Ile Trp Asp Asn Glu Thr Pro Ile Cys Asp Arg Ile Pro Cys Gly Leu
Pro Pro Thr Ile Thr Asn Gly Asp Phe Ile Ser Thr Asn Arg Glu Asn
Phe His Tyr Gly Ser Val Val Thr Tyr Arg Cys Asn Pro Gly Ser Gly
Gly Arg Lys Val Phe Glu Leu Val Gly Glu Pro Ser Ile Tyr Cys Thr
Ser Asn Asp Asp Gln Val Gly Ile Trp Ser Gly Pro Ala Pro Gln Cys
Ile Ile Pro Asn Lys Asp Gly Pro Lys Lys Lys Lys Lys Lys Ser Pro

-> <-
Ser Lys Ser Ser Gly Cys-S-S-{Cys Asp Gly Pro Lys Lys Lys Lys Lys
| |
CO_2H $CONH_2$
Lys Ser Pro Ser Lys Ser Ser Gly} (N-Myristoyl)

SEQ ID NO 10

linear, 2 polypeptide chains disulphide linked

1 [QCNAPEWLPF ARPTNLTDEF EFPIGTYLNY ECRPGYSGRP
41 FSIICLKNSV WTGAKDRCRR KSCRNPPDPV NGMVHVIKGI
81 QFGSQIKYSC TKGYRLIGSS SATCIISGDT VIWDNETPIC
121 DRIPCGLPPT ITNGDFISTN RENFHYGSVV TYRCNPGSGG
161 RKVFELVGEP SIYCTSNDDQ VGIWSGPAPQ CIIPNKCTPP
201 NVENGILVSD NRSLFSLNEV VEFRCQPGFV MKGPRRVKCQ
241 ALNKWEPELP SCSRVCQPPP DVLHAERTQR DKDNFSPGQE
281 VFYSCEPGYD LRGAASMRCT PQGDWSPAAP TCEVKSCDDF
321 MGQLLNGRVL FPVNLQLGAK VDFVCDEGFQ LKGSSASYCV
361 LAGMESLWNS SVPVCEQIFC PSPPVIPNGR HTGKPLEVFP
401 FGKAVNYTCD PHPDRGTSFD LIGESTIRCT SDPQGNGVWS
441 SPAPRCGILG HCQAPDHFLF AKLKTQTNAS DFPIGTSLKY
481 ECRPEYYGRP FSITCLDNLV WSSPKDVCKR KSCKTPPDPV
521 NGMVHVITDI QVGSRINYSC TTGHRLIGHS SAECILSGNA
561 AHWSTKPPIC QRIPCGLPPT IANGDFISTN RENFHYGSVV
601 TYRCNPGSGG RKVFELVGEP SIYCTSNDDQ VGIWSGPAPQ
641 CIIPNKCTPP NVENGILVSD NRSLFSLNEV VEFRCQPGFV

681 MKGPRRVKCQ ALNKWEPELP SCSRVCQPPP DVLHAERTQR
721 DKDNFSPGQE VFYSCEPGYD LRGAASMRCT PQGDWSPAAP
761 TCEVKSCDDF MGQLLNGRVL FPVNLQLGAK VDFVCDEGFQ
801 LKGSSASYCV LAGMESLWNS SVPVCEQIFC PSPPVIPNGR
841 HTGKPLEVFP FGKAVNYTCD PHPDRGTSFD LIGESTIRCT
881 SDPQGNGVWS SPAPRCGILG HCQAPDHFLF AKLKTQTNAS
921 DFPIGTSLKY ECRPEYYGRP FSITCLDNLV WSSPKDVCKR
961 KSCKTPPDPV NGMVHVITDI QVGSRINYSC TTGHRLIGHS
1001 SAECILSGNT AHWSTKPPIC QRIPCGLPPT IANGDFISTN
1041 RENFHYGSVV TYRCNLGSRG RKVFELVGEP SIYCTSNDDQ
1081 VGIWSGPAPQ CIIPNKCTPP NVENGILVSD NRSLFSLNEV
1121 VEFRCQPGFV MKGPRRVKCQ ALNKWEPELP SCSRVCQPPP
1161 EILHGEHTPS HQDNFSPGQE VFYSCEPGYD LRGAASLHCT
1201 PQGDWSPEAP RCAVKSCDDF LGQLPHGRVL FPLNLQLGAK
1241 VSFVCDEGFR LKGSSVSHCV LVGMRSLWNN SVPVCEHIFC
1281 PNPPAILNGR HTGTPSGDIP YGKEISYTCD PHPDRGMTFN
1321 LIGESTIRCT SDPHGNGVWS SPAPRCELSV RAGHCKTPEQ
1361 FPFASPTIPI NDFEFPVGTS LNYECRPGYF GKMFSISCLE
1401 NLVWSSVEDN CRRKSCGPPP EPFNGMVHIN TDTQFGSTVN
1441 YSCNEGFRLI GSPSTTCLVS GNNVTWDKKA PICEIISCEP
1481 PPTISNGDFY SNNRTSFHNG TVVTYQCHTG PDGEQLFELV
1521 GERSIYCTSK DDQVGVWSSP PPRCISTNKC TAPEVENAIR
1561 VPGNRSFFSL TEIIRFRCQP GFVMVGSHTV QCQTNGRWGP
1601 KLPHCSRVCQ PPPEILHGEH TLSHQDNFSP GQEVFYSCEP
1641 SYDLRGAASL HCTPQGDWSP EAPRCTVKSC DDFLGQLPHG
1681 RVLLPLNLQL GAKVSFVCDE GFRLKGRSAS HCVLAGMKAL
1721 WNSSVPVCEQ IFCPNPPAIL NGRHTGTPFG DIPYGKEISY
1761 ACDTHPDRGM TFNLIGESSI RCTSDPQGNG VWSSPAPRCE
1801 LSVPAACPHP PKIQNGHYIG GHVSLYLPGM TISYTCDPGY
1841 LLVGKGFIFC TDQGIWSQLD HYCKEVNCSF PLFMNGISKE
1881 LEMKKVYHYG DYVTLKCEDG YTLEGSPWSQ CQADDRWDPP

-> <-
1921 LAKCTSRAHC]-S-S-{CDGPKKKKKKSPSKSSG}-(N-Myristoyl)
| |
CO_2H $CONH_2$

In SEQ ID NO 10, peptide sequences are given in brackets in single letter amino acid code.

SEQ ID No. 11

linear, 2 polypeptide chains disulphide linked

Met Gln Cys Asn Ala Pro Glu Trp Leu Pro Phe Ala Arg Pro Thr Asn
Leu Thr Asp Glu Phe Glu Phe Pro Ile Gly Thr Tyr Leu Asn Tyr Glu
Cys Arg Pro Gly Tyr Ser Gly Arg Pro Phe Ser Ile Ile Cys Leu Lys
Asn Ser Val Trp Thr Gly Ala Lys Asp Arg Cys Arg Arg Lys Ser Cys
Arg Asn Pro Pro Asp Pro Val Asn Gly Met Val His Val Ile Lys Gly
Ile Gln Phe Gly Ser Gln Ile Lys Tyr Ser Cys Thr Lys Gly Tyr Arg
Leu Ile Gly Ser Ser Ser Ala Thr Cys Ile Ile Ser Gly Asp Thr Val
Ile Trp Asp Asn Glu Thr Pro Ile Cys Asp Arg Ile Pro Cys Gly Leu
Pro Pro Thr Ile Thr Asn Gly Asp Phe Ile Ser Thr Asn Arg Glu Asn
Phe His Tyr Gly Ser Val Val Thr Tyr Arg Cys Asn Pro Gly Ser Gly
Gly Arg Lys Val Phe Glu Leu Val Gly Glu Pro Ser Ile Tyr Cys Thr
Ser Asn Asp Asp Gln Val Gly Ile Trp Ser Gly Pro Ala Pro Gln Cys
Ile Ile Pro Asn Lys Cys-S-S-Cys-Asp-Gly-Pro-Lys-Lys-Lys-Lys-Lys-
|
$NHCOCH_3$
Lys-Ser-Pro-Ser Lys-Ser-Ser-Lys-(N-Myristoyl)-NH_2

SEQ ID No. 12

linear, 2 polypeptide chains disulphide linked

Met Gln Cys Asn Ala Pro Glu Trp Leu Pro Phe Ala Arg Pro Thr Asn
Leu Thr Asp Glu Phe Glu Phe Pro Ile Gly Thr Tyr Leu Asn Tyr Glu
Cys Arg Pro Gly Tyr Ser Gly Arg Pro Phe Ser Ile Ile Cys Leu Lys
Asn Ser Val Trp Thr Gly Ala Lys Asp Arg Cys Arg Arg Lys Ser Cys
Arg Asn Pro Pro Asp Pro Val Asn Gly Met Val His Val Ile Lys Gly
Ile Gln Phe Gly Ser Gln Ile Lys Tyr Ser Cys Thr Lys Gly Tyr Arg
Leu Ile Gly Ser Ser Ser Ala Thr Cys Ile Ile Ser Gly Asp Thr Val
Ile Trp Asp Asn Glu Thr Pro Ile Cys Asp Arg Ile Pro Cys Gly Leu
Pro Pro Thr Ile Thr Asn Gly Asp Phe Ile Ser Thr Asn Arg Glu Asn
Phe His Tyr Gly Ser Val Val Thr Tyr Arg Cys Asn Pro Gly Ser Gly
Gly Arg Lys Val Phe Glu Leu Val Gly Glu Pro Ser Ile Tyr Cys Thr
Ser Asn Asp Asp Gln Val Gly Ile Trp Ser Gly Pro Ala Pro Gln Cys
-> <-
Ile Ile Pro Asn Lys Cys-S-S-{Cys-Lys-Thr-Lys-Ser-Lys-Lys-Lys-Lys-
|
$CONH_2$
Lys-Lys-Gly-Asp-Lsy-Ser)-NH-(Myristoyl)

SEQ ID No. 13

linear, 2 polypeptide chains disulphide linked

Met Gln Cys Asn Ala Pro Glu Trp Leu Pro Phe Ala Arg Pro Thr Asn
Leu Thr Asp Glu Phe Glu Phe Pro Ile Gly Thr Tyr Leu Asn Tyr Glu
Cys Arg Pro Gly Tyr Ser Gly Arg Pro Phe Ser Ile Ile Cys Leu Lys
Asn Ser Val Trp Thr Gly Ala Lys Asp Arg Cys Arg Arg Lys Ser Cys
Arg Asn Pro Pro Asp Pro Val Asn Gly Met Val His Val Ile Lys Gly
Ile Gln Phe Gly Ser Gln Ile Lys Tyr Ser Cys Thr Lys Gly Tyr Arg
Leu Ile Gly Ser Ser Ser Ala Thr Cys Ile Ile Ser Gly Asp Thr Val
Ile Trp Asp Asn Glu Thr Pro Ile Cys Asp Arg Ile Pro Cys Gly Leu
Pro Pro Thr Ile Thr Asn Gly Asp Phe Ile Ser Thr Asn Arg Glu Asn
Phe His Tyr Gly Ser Val Val Thr Tyr Arg Cys Asn Pro Gly Ser Gly
Gly Arg Lys Val Phe Glu Leu Val Gly Glu Pro Ser Ile Tyr Cys Thr
Ser Asn Asp Asp Gln Val Gly Ile Trp Ser Gly Pro Ala Pro Gln Cys
Ile Ile Pro Asn Lys Cys-S-S-Cys-Ser-Ala-Ala-Pro-Ser-Ser-Gly-Phe-
                          |
                        $NHCOCH_3$
Arg-Ile-Leu-Leu-Leu-Lys-Val-$NH(CH_2)_9CH_3$

SEQ ID NO:14

Met Gln Cys Asn Ala Pro Glu Trp Leu Pro Phe Ala Arg Pro Thr Asn
Leu Thr Asp Glu Phe Glu Phe Pro Ile Gly Thr Tyr Leu Asn Tyr Glu
Cys Arg Pro Gly Tyr Ser Gly Arg Pro Phe Ser Ile Ile Cys Leu Lys
Asn Ser Val Trp Thr Gly Ala Lys Asp Arg Cys Arg Arg Lys Ser Cys
Arg Asn Pro Pro Asp Pro Val Asn Gly Met Val His Val Ile Lys Gly
Ile Gln Phe Gly Ser Gln Ile Lys Tyr Ser Cys Thr Lys Gly Tyr Arg
Leu Ile Gly Ser Ser Ser Ala Thr Cys Ile Ile Ser Gly Asp Thr Val
Ile Trp Asp Asn Glu Thr Pro Ile Cys Asp Arg Ile Pro Cys Gly Leu
Pro Pro Thr Ile Thr Asn Gly Asp Phe Ile Ser Thr Asn Arg Glu Asn
Phe His Tyr Gly Ser Val Val Thr Tyr Arg Cys Asn Pro Gly Ser Gly
Gly Arg Lys Val Phe Glu Leu Val Gly Glu Pro Ser Ile Tyr Cys Thr
Ser Asn Asp Asp Gln Val Gly Ile Trp Ser Gly Pro Ala Pro Gln Cys
Ile Ile Pro Thr Asn Ala Asn Lys Ser Leu Ser Ser Ile Ser Cys Gln
Thr

SEQ ID No 15

CTGGAGCGGGCCCGCACCGCAGTGCATCATCCCGAACAAATGCTAATAAAAGC

SEQ ID No 16

GCTTTTATTAGCATTTGTTCGGGATGATGCACTGCGGTGCGGGCCCGCTCCAG

SEQ ID No 17

linear, 2 polypeptide chains disulphide linked

Met Gln Cys Asn Ala Pro Glu Trp Leu Pro Phe Ala Arg Pro Thr Asn
Leu Thr Asp Glu Phe Glu Phe Pro Ile Gly Thr Tyr Leu Asn Tyr Glu
Cys Arg Pro Gly Tyr Ser Gly Arg Pro Phe Ser Ile Ile Cys Leu Lys
Asn Ser Val Trp Thr Gly Ala Lys Asp Arg Cys Arg Arg Lys Ser Cys
Arg Asn Pro Pro Asp Pro Val Asn Gly Met Val His Val Ile Lys Gly
Ile Gln Phe Gly Ser Gln Ile Lys Tyr Ser Cys Thr Lys Gly Tyr Arg
Leu Ile Gly Ser Ser Ser Ala Thr Cys Ile Ile Ser Gly Asp Thr Val
Ile Trp Asp Asn Glu Thr Pro Ile Cys Asp Arg Ile Pro Cys Gly Leu
Pro Pro Thr Ile Thr Asn Gly Asp Phe Ile Ser Thr Asn Arg Glu Asn
Phe His Tyr Gly Ser Val Val Thr Tyr Arg Cys Asn Pro Gly Ser Gly
Gly Arg Lys Val Phe Glu Leu Val Gly Glu Pro Ser Ile Tyr Cys Thr
Ser Asn Asp Asp Gln Val Gly Ile Trp Ser Gly Pro Ala Pro Gln Cys

Ile Ile Pro Thr Asn Ala Asn Lys Ser Leu Ser Ser Ile Ser Cys-S-S-{Cys-
                                                  -> <-
                                                  Gln-Thr CONH$_2$

-Asp-Gly-Pro-Lys-Lys-Lys-Lys-Lys-Lys-Ser-Lys-Ser-Ser-Gly}-NH-(Myristoyl)

SEQ ID No 18

Cys-Asp-Gly-Pro-Lys-Lys-Lys-Lys-Lys-Lys-Ser-Pro-Ser-Lys-Ser-Ser-Lys-NH$_2$

SEQ ID No 19

Ser-Lys-Asp-Gly-Lys-Lys-Lys-Lys-Lys-Lys-Ser-Lys-Thr-Lys-Cys

SEQ ID No 20

Cys-Ser-Ala-Ala-Pro-Ser-Ser-Gly-Phe-Arg-Ile-Leu-Leu-Leu-Lys-Val

SEQ ID No 21

linear, 2 polypeptide chains disulphide linked

N-(myristoyl)-Gly-Ser-Ser-Lys-Ser-Pro-Ser-Lys-Lys-Lys-Lys-Lys-Lys-Pro-
Gly-Asp-Cys-NH$_2$
|
S-S-[4-butyrimino]-N-ε(Lys)[Streptokinase]

SEQ ID No 22

Single chain form of the 527 amino acid residue intact t-PA molecule.

Residue 478 (serine) has been modified as shown below

[SYQVICRDEKTQMIYQQHQSWLRPVLRSNRVEYCWCNSGRAQCHSVPVKSCSEPRCFN

GGTCQQALYFSDFVCQCPEGFAGKCCEIDTRATCYEDQGISYRGTWSTAESGAECTNW

NSSALAQKPYSGRRPDAIRLGLGNHNYCRNPDRDSKPWCYVFKAGKYSSEFCSTPACS

EGNSDCYFGNGSAYRGTHSLTESGASCLPWNSMILIGKVYTAQNPSAQALGLGKHNYC

RNPDGDAKPWCHVLKNRRLTWEYCDVPSCSTCGLRQYSQPQFRIKGGLFADIASHPWQA

AIFAKHRRSPGERFLCGGILISSCWILSAAHCFQERFPPHHLTVILGRTYRVVPGEE

EQKFEVEKYIVHKEFDDDTYDNDIALLQLKSDSSRCAQESSVVRTVCLPPADLQLPDW

TECELSGYGKHEALSPFYSERLKEAHVRLYPSSRCTSQHLLNRTVTDNMLCAGDTRSG

GPQANLHDACQGDSGGPLVCLNDGRMTLVGIISWGLGCGQKDVPGVYTKVTNYLDWIRDNMRP]

<-

Ser 478 O-4-CO-benzyl-NH$(CH_2)_2$NHCO$(CH_2)_2$-S-S-(Cys-Asp-Gly-Pro-Lys-Lys-

|

$CONH_2$

Lys-Lys-Lys-Lys-Ser-Pro-Ser-Lys-Ser-Ser-Gly)-NH-(Myristoyl)

SEQ ID No 23

Met Gln Cys Asn Ala Pro Glu Trp Leu Pro Phe Ala Arg Pro Thr Asn
Leu Thr Asp Glu Phe Glu Phe Pro Ile Gly Thr Tyr Leu Asn Tyr Glu
Cys Arg Pro Gly Tyr Ser Gly Arg Pro Phe Ser Ile Ile Cys Leu Lys
Asn Ser Val Trp Thr Gly Ala Lys Asp Arg Cys Arg Arg Lys Ser Cys
Arg Asn Pro Pro Asp Pro Val Asn Gly Met Val His Val Ile Lys Gly
Ile Gln Phe Gly Ser Gln Ile Lys Tyr Ser Cys Thr Lys Gly Tyr Arg
Leu Ile Gly Ser Ser Ser Ala Thr Cys Ile Ile Ser Gly Asp Thr Val
Ile Trp Asp Asn Glu Thr Pro Ile Cys Asp Arg Ile Pro Cys Gly Leu
Pro Pro Thr Ile Thr Asn Gly Asp Phe Ile Ser Thr Asn Arg Glu Asn
Phe His Tyr Gly Ser Val Val Thr Tyr Arg Cys Asn Pro Gly Ser Gly
Gly Arg Lys Val Phe Glu Leu Val Gly Glu Pro Ser Ile Tyr Cys Thr
Ser Asn Asp Asp Gln Val Gly Ile Trp Ser Gly Pro Ala Pro Gln Cys
Ile Ile Pro Asn Lys Asp Gly Pro Ser Glu Ile Leu Arg Gly Asp Phe
Ser Ser Cys

SEQ ID No 24

linear, 2 polypeptide chains disulphide linked

Met Gln Cys Asn Ala Pro Glu Trp Leu Pro Phe Ala Arg Pro Thr Asn
Leu Thr Asp Glu Phe Glu Phe Pro Ile Gly Thr Tyr Leu Asn Tyr Glu
Cys Arg Pro Gly Tyr Ser Gly Arg Pro Phe Ser Ile Ile Cys Leu Lys

Asn Ser Val Trp Thr Gly Ala Lys Asp Arg Cys Arg Arg Lys Ser Cys
Arg Asn Pro Pro Asp Pro Val Asn Gly Met Val His Val Ile Lys Gly
Ile Gln Phe Gly Ser Gln Ile Lys Tyr Ser Cys Thr Lys Gly Tyr Arg
Leu Ile Gly Ser Ser Ser Ala Thr Cys Ile Ile Ser Gly Asp Thr Val
Ile Trp Asp Asn Glu Thr Pro Ile Cys Asp Arg Ile Pro Cys Gly Leu
Pro Pro Thr Ile Thr Asn Gly Asp Phe Ile Ser Thr Asn Arg Glu Asn
Phe His Tyr Gly Ser Val Val Thr Tyr Arg Cys Asn Pro Gly Ser Gly
Gly Arg Lys Val Phe Glu Leu Val Gly Glu Pro Ser Ile Tyr Cys Thr
Ser Asn Asp Asp Gln Val Gly Ile Trp Ser Gly Pro Ala Pro Gln Cys
Ile Ile Pro Asn Lys Asp-Gly-Pro-Ser-Glu-Ile-Leu-Arg-Gly Asp-Phe-
<-
Ser-Ser-Cys-S-S-(Cys-Asp-Gly-Pro-Lys-Lys-Lys-Lys Lys-Lys-Ser-Pro-Ser-
Lys-Ser-Ser-Gly)-NH-(Myristoyl)

SEQ ID No 25
CGCACCGCAGTGCATCATCCCGAACAAAGATGGCCCGAGCGAAATTCTGCGTGGCGATTTTAGCAGCTGCTA

SEQ ID No 26
ACGTTAGCAGCTGCTAAAATCGCCACGCAGAATTTCGCTCGGGCCATCTTTGTTCGGGATGATGCACTGCGG
TGCGGGCC

SEQ ID No 27
N-(Myristoyl)-Gly-Ser-Ser-Lys-Ser-Pro-Ser-Lys-Lys-Lys-Lys-Lys-Lys-Pro-
Gly-Asp-(S-2-thiopyridyl)Cys-NH_2

SEQ ID No 28
N-acetyl-(S-2-thiopyridyl)Cys Asp-Gly-Pro-Lys-Lys-Lys-Lys-Lys-Lys-Ser-
Pro-Ser Lys-Ser-Ser-(εN-(Myristoyl))Lys-NH_2

SEQ ID No 29
N-(Myristoyl)-Ser-Lys-Asp-Gly-Lys-Lys-Lys-Lys-Lys-Lys-Ser-Lys-Thr-Lys-
(S-2-Thiopyridyl)Cys-NH_2

SEQ ID No 30
N-acetyl-(S-2-thiopyridyl)Cys-Ser-Ala-Ala-Pro-Ser-Ser-Gly-Phe-Arg-Ile-
Leu-Leu-Leu-Lys-Val-$NH(CH_2)_9CH_3$

SEQ ID No 31

Met Gln Cys Asn Ala Pro Glu Trp Leu Pro Phe Ala Arg Pro Thr Asn
Leu Thr Asp Glu Phe Glu Phe Pro Ile Gly Thr Tyr Leu Asn Tyr Glu
Cys Arg Pro Gly Tyr Ser Gly Arg Pro Phe Ser Ile Ile Cys Leu Lys
Asn Ser Val Trp Thr Gly Ala Lys Asp Arg Cys Arg Arg Lys Ser Cys
Arg Asn Pro Pro Asp Pro Val Asn Gly Met Val His Val Ile Lys Gly
Ile Gln Phe Gly Ser Gln Ile Lys Tyr Ser Cys Thr Lys Gly Tyr Arg
Leu Ile Gly Ser Ser Ser Ala Thr Cys Ile Ile Ser Gly Asp Thr Val
Ile Trp Asp Asn Glu Thr Pro Ile Cys Asp Arg Ile Pro Cys Gly Leu
Pro Pro Thr Ile Thr Asn Gly Asp Phe Ile Ser Thr Asn Arg Glu Asn
Phe His Tyr Gly Ser Val Val Thr Tyr Arg Cys Asn Pro Gly Ser Gly
Gly Arg Lys Val Phe Glu Leu Val Gly Glu Pro Ser Ile Tyr Cys Thr
Ser Asn Asp Asp Gln Val Gly Ile Trp Ser Gly Pro Ala Pro Gln Cys
Ile Ile Pro Asn Lys Asp Gly Pro Lys Lys Lys Lys Lys Lys Ser Pro
Ser Lys Ser Ser Gly Cys-S-S-$(CH_2)_2$-CONH-$(CH_2)_{12}CH_3$

SEQ ID No 32

linear, 2 polypeptide chains disulphide linked

N-(myristoyl)-Gly-Ser-Ser-Lys-Ser-Pro-Ser-Lys-Lys-Lys-Lys-Lys-Lys-Pro-Gly-Asp-Cys-NH_2

S-S-[4-butyrimino]-N-ε(Lys)[Rabbit anti-(human erythrocyte membrane) antibody]

SEQ ID No 33

Met Gln Cys Asn Ala Pro Glu Trp Leu Pro Phe Ala Arg Pro Thr Asn
Leu Thr Asp Glu Phe Glu Phe Pro Ile Gly Thr Tyr Leu Asn Tyr Glu
Cys Arg Pro Gly Tyr Ser Gly Arg Pro Phe Ser Ile Ile Cys Leu Lys
Asn Ser Val Trp Thr Gly Ala Lys Asp Arg Cys Arg Arg Lys Ser Cys
Arg Asn Pro Pro Asp Pro Val Asn Gly Met Val His Val Ile Lys Gly
Ile Gln Phe Gly Ser Gln Ile Lys Tyr Ser Cys Thr Lys Gly Tyr Arg
Leu Ile Gly Ser Ser Ser Ala Thr Cys Ile Ile Ser Gly Asp Thr Val
Ile Trp Asp Asn Glu Thr Pro Ile Cys Asp Arg Ile Pro Cys Gly Leu
Pro Pro Thr Ile Thr Asn Gly Asp Phe Ile Ser Thr Asn Arg Glu Asn
Phe His Tyr Gly Ser Val Val Thr Tyr Arg Cys Asn Pro Gly Ser Gly
Gly Arg Lys Val Phe Glu Leu Val Gly Glu Pro Ser Ile Tyr Cys Thr
Ser Asn Asp Asp Gln Val Gly Ile Trp Ser Gly Pro Ala Pro Gln Cys
Ile Ile Pro Asn Lys Ala Ala Pro Ser Val Ile Gly Phe Arg Ile Leu
Leu Leu Lys Val Gly Cys

SEQ ID No 34

CGCACCGCAGTGCATCATCCCGAACAAAGCGGCGCCCAGCGTGATTGGCTTCCGTATTCTGCTGCTGAAAGT
GGCGGGCTGCTA

SEQ ID No 35

AGCTTAGCAGCCCGCCACTTTCAGCAGCAGAATACGGAAGCCAATCACGCTGGGCGCCGCTTTGTTCGGGAT
GATGCACTGCGGTGCGGGCC

SEQ ID No 36

linear, 2 polypeptide chains disulphide linked

Met Gln Cys Asn Ala Pro Glu Trp Leu Pro Phe Ala Arg Pro Thr Asn
Leu Thr Asp Glu Phe Glu Phe Pro Ile Gly Thr Tyr Leu Asn Tyr Glu
Cys Arg Pro Gly Tyr Ser Gly Arg Pro Phe Ser Ile Ile Cys Leu Lys
Asn Ser Val Trp Thr Gly Ala Lys Asp Arg Cys Arg Arg Lys Ser Cys
Arg Asn Pro Pro Asp Pro Val Asn Gly Met Val His Val Ile Lys Gly
Ile Gln Phe Gly Ser Gln Ile Lys Tyr Ser Cys Thr Lys Gly Tyr Arg
Leu Ile Gly Ser Ser Ser Ala Thr Cys Ile Ile Ser Gly Asp Thr Val
Ile Trp Asp Asn Glu Thr Pro Ile Cys Asp Arg Ile Pro Cys Gly Leu
Pro Pro Thr Ile Thr Asn Gly Asp Phe Ile Ser Thr Asn Arg Glu Asn
Phe His Tyr Gly Ser Val Val Thr Tyr Arg Cys Asn Pro Gly Ser Gly
Gly Arg Lys Val Phe Glu Leu Val Gly Glu Pro Ser Ile Tyr Cys Thr
Ser Asn Asp Asp Gln Val Gly Ile Trp Ser Gly Pro Ala Pro Gln Cys
Ile Ile Pro Asn Lys Ala Ala Pro Ser Val Ile Gly Phe Arg Ile Leu
<-
Leu Leu Lys Val Gly Cys S-S-{Cys-Asp-Gly-Pro-Lys-Lys-Lys-Lys Lys-Lys-
Ser-Pro-Ser-Lys-Ser-Ser-Gly}-NH-(Myristoyl)

CLAIMS

1. A soluble derivative of a soluble polypeptide, said derivative comprising two or more heterologous membrane binding elements with low membrane affinity covalently associated with the polypeptide which elements are capable of interacting, independently and with thermodynamic additivity, with components of cellular or artificial membranes exposed to extracellular fluids.

2. A derivative according to claim 1 wherein each membrane binding element with low membrane affinityhas a dissociation constant 1μM-1mM.

3. A derivative according to claim 1 or 2 wherein each membrane binding element has a size <5kDa.

4. A derivative according to any preceding claim which incorporates sufficient elements with low affinities for membrane components to result in a 0.01 - 10nM dissociation constant affinity for specific membranes.

5. A derivative according to any preceding claim which has a solubility in pharmaceutial formulation media >100μg/ml.

6. A derivative according to any preceding claim wherein at least one element is hydrophilic.

7. A derivative according to any preceding claim which comprises two to eight membrane binding elements.

8. A derivative according to any preceding claim wherein the membrane binding elements are selected from: fatty acid derivatives; basic amino acid sequences; ligands of known integral membrane proteins; sequences derived from the complementarity-determining region of monoclonal antibodies raised against epitopes of membrane proteins; and membrane binding sequences identified through screening of random chemical or peptide libraries.

9. A derivative according to claim 8 wherein a membrane binding element is a fatty acid derivative selected from aliphatic acyl groups with about 8 to 18 methylene units, long-chain (8-18 methylene) aliphatic amines and thiols, steroid and farnesyl derivatives.

10. A derivative according to claim 8 or 9 wherein a membrane binding element is a basic aminoacid sequence including (Lys)n where n is from 3 to 10.

11. A derivative according to claim 10 wherein the amino acid sequence is selected from:

i) DGPKKKKKKSPSKSSG

ii) GSSKSPSKKKKKKPGD

iii) SPSNETPKKKKKRFSFKKSG

iv) DGPKKKKKKSPSKSSK

v) SKDGKKKKKKSKTK

(N-terminus on left)

12. A derivative according to any of claims 8 to 10 wherein a membrane binding element is a ligand of a known integral membrane protein selected from GRGDSP, DGPSEILRGDFSS, GNEQSFRVDLRTLLRYA, GFRILLLKV, SAAPSSGFRILLLKV and AAPSVIGFRILLLKVAG or the carbohydrate ligand Sialyl Lewis^x.

13. A derivative according to any preceding claim wherein the soluble polypeptide is selected from IL-4 Y124D mutein, prourokinase, streptokinase, tissue-type plasminogen activator, reteplase, leptin, complement inhibitors selected from complement regulatory proteins and hybrids or muteins thereof, scFv antibody against cytokines, Protein C, antibodies against CD4, B7/CD28, CD3/TCR or CD11b(CR3) and Interferon-β and derivatives.

14. A derivative according to any preceding claim which has the following structure:

$$[P]\text{-}\{L\text{-}[W]\}_n\text{-}X$$

in which:

P is the soluble polypeptide,

each **L** is independently a flexible linker group,

each **W** is independently a peptidic membrane binding element,

n is an integer of 1 or more and

X is a peptidic or non-peptidic membrane-binding entity which may be covalently linked to any **W**.

15. A derivative according to claim 14 wherein peptidic membrane binding elements are 8 to 20 amino acids long and elements **W** are located sequentially either at the N or C terminus of the soluble polypeptide and the amino acid sequences are linked to one

another and to the soluble peptide by linker groups which are selected from: hydrophilic and/or flexible aminoacid sequences of 4 to 20 aminoacids; linear hydrophilic synthetic polymers; and chemical bridging groups.

16. A derivative according to claim 14 or 15 wherein the chemical bridging groups are of formula (I):

-A-R-B- (I)

in which each of A and B, which may be the same or different, represents -CO-, $-C(=NH_2^+)-$, maleimido, -S- or a bond and R is a bond or a linking group containing one or more $-(CH_2)-$ or meta-, ortho- or para- disubstituted phenyl units optionally together with a hydrophilic portion.

17. A derivative according to claim 16 wherein R is selected from $-(CH_2)_r-$, $-(CH_2)_p-S-S-(CH_2)_q-$ and $-(CH_2)_p-CH(OH)-CH(OH)-(CH_2)_q-$, in which r is an integer of at least 2, and p and q are independently integers of at least 2, or $(CH_2)_2CONH(CH_2)_nNH$-(4-phenyl) where n is an integer of 3 to 8.

18. A soluble derivative according to claim 1 of SEQ ID NO: 32.

19. A soluble derivative according to any of claims 1 to 17 wherein the soluble polypeptide is a soluble complement inhibitor.

20. A soluble derivative according to claim 19 wherein the soluble polypeptide is a soluble CR1 polypeptide fragment.

21. A soluble derivative according to claim 20 wherein the soluble CR1 polypeptide consists of residues 1-196 of CR1 and with an N-terminal methionine and the derivative comprises a myristoyl group and one or more polypeptides sequence selected from

DGPKKKKKKSPSKSSGC
GSSKSPSKKKKKKPGDC
CDGPKKKKKKSPSKSSK
SKDGKKKKKKSKTKC
CSAAPSSGFRILLLKV
AAPSVIGFRILLLKVAGC
and
DGPSEILRGDFSSC
(N-terminus on left).

22. A soluble derivative according to claim 20 or 21 selected from SEQ ID NOs: 8, 31, 9, 10, 11, 12, 13, 17, 24 and 36.

23. A soluble derivative according to any of claims 1 to 17 wherein the soluble polypeptide is a thrombolytic agent.

24. A soluble derivative according to claim 23 selected from SEQ ID NOs: 21 and 22.

25. A process for preparing a derivative according to claim 1 which process comprises expressing DNA encoding the polypeptide portion of said derivative in a recombinant host cell and recovering the product and thereafter post translationally modifying the polypeptide to chemically introduce membrane binding elements.

26. A polypeptide portion of a derivative according to claim 1, comprising the soluble peptide linked by a peptide bond to one peptidic membrane binding element

27. A soluble polypeptide including a C-terminal cysteine.

28. The polypeptide of SEQ ID NO: 7, 23, 33, 6 or 14.

29. A DNA polymer encoding the polypeptide portion of claim 26, 27 or 28.

30. A replicable expression vector capable, in a host cell, of expressing the DNA polymer of claim 29.

31. A host cell transformed with a replicable expression vector of claim 30.

32. A peptide membrane binding element with low membrane affinity comprising one or more derivatisations selected from:
a terminal cysteine residue optionally activated at the thiol group;
an N-haloacetyl group (where halo signifies chlorine, bromine or iodine) located at the N-terminus of the the peptide or at an ε-amino group of a lysine residue;
an amide group at the C-terminus;
an N-terminal blocking group; and
a fatty acid N-acyl group at the N-terminus or at an ε-amino group of a lysine residue.

33. A peptidic membrane binding element derivatised according to claim 32 wherein the peptide has the amino sequence of a peptide defined in claim 11 or 12 and a fatty acid N-acyl group of 8 to 18 methylene units at the N-terminus or at an ε-amino group of a lysine residue of the peptide.

34. A peptidic membrane binding element derivatised according to claim 32 or 33 selected from SEQ ID Nos: 27, 28, 29 and 30.

35. A C_{10-20} fatty acyl derivative of an aminoC_{2-6}alkane thiol (optionally C-substituted).

36. A compound according to claim 35 selected from N-(2-myristoyl) aminoethanethiol nd N-myristoyl L-cysteine.

37. A pharmaceutical composition comprising a derivative according to any of claims 1 to 24 in combination with a pharmaceutically acceptable carrier.

38. A derivative according to any of claims 1 to 24 for use as a medicament.

39. A method of treatment of disorders amenable to treatment by a soluble peptide which comprises administering a soluble derivative of said soluble peptide according to any of claims 1 to 24.

40. The use of a derivative according to any of claims 1 to 24 for the preparation of a medicament for treatment of disorders amenable to treatment by the soluble peptide.

41. A pharmaceutical composition for treating a disease or disorder associated with inflammation or inappropriate complement activation comprising a therapeutically effective amount of a derivative according to claim 19 and a pharmaceutically acceptable carrier or excipient.

42. A method of treating a disease or disorder associated with inflammation or inappropriate complement activation comprising administering to a subject in need of such treatment a therapeutically effective amount of a derivative according to claim 19.

43. The use of a derivative of claim 19 in the manufacture of a medicament for the treatment of a disease or disorder associated with inflammation or inappropriate complement activation.

44. A pharmaceutical composition for treating a disease or disorder associated with inflammation or inappropriate complement activation comprising a therapeutically effective amount of a soluble CR1 polypeptide derivative according to any of claims 20 to 22, and a pharmaceutically acceptable carrier or excipient.

45. A method of treating a disease or disorder associated with inflammation or inappropriate complement activation comprising administering to a subject in need of such treatment a therapeutically effective amount of a a soluble CR1 polypeptide derivative of any one of claims 20 to 22.

46. The use of a soluble CR1 polypeptide derivative of any one of claims 20 to 22 in the manufacture of a medicament for the treatment of a disease or disorder associated with inflammation or inappropriate complement activation.

47. A pharmaceutical composition for treating thrombotic disorders comprising a therapeutically effective amount of a derivative according to claim 23 or 24 and a pharmaceutically acceptable carrier or excipient.

48. A method of treating thrombotic disorders comprising administering to a subject in need of such treatment a therapeutically effective amount of a derivative according to claim 23 or 24.

49. The use of a derivative according to claim 23 or 24 in the manufacture of a medicament for the treatment of thrombotic disorders.

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(71) Applicant *(for all designated States except US)*: WASHINGTON UNIVERSITY [US/US]; One Brookings Drive, St. Louis, MO 63130 (US).

(72) Inventor; and
(75) Inventor/Applicant *(for US only)*: GROSS, Richard, W. [US/US]; 307 Chesterfield Oaks, St. Louis, MO 63005 (US).

(74) Agent: DEHLINGER, Peter, J.; Dehlinger & Associates, P.O. Box 60850, Palo Alto, CA 94306-0850 (US).

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(54) Title: CELL MEMBRANE FUSION COMPOSITION AND METHOD

(57) Abstract

A lipid vesicle composition for use in delivering a vesicle-encapsulated agent to a target cell is disclosed. The composition is formed of vesicle-forming lipids, including at least 10 mole percent plasmalogen phospholipid with a small-volume polar head group. The composition may also include a fusion protein for promoting fusion of the vesicles to the target cells. A novel fusion protein identified as an isoform of glyceraldehyde-3-phosphate dehydrogenase is also disclosed.

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CELL MEMBRANE FUSION COMPOSITION AND METHOD

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1. Field of the Invention

The present invention relates to fusogenic lipid vesicles, and to the use of such vesicles, *e.g.*, for drug delivery.

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3. Background of the Invention

One of the limiting factors in the pharmacokinetic behavior of many therapeutic drugs is drug uptake by target cells. For many small, uncharged drug compounds, drug permeation across the cell membrane may allow relatively efficient drug uptake by the cell. However, for a variety of larger and/or charged compounds, such as proteins, nucleic acids, and highly water soluble charged organic compounds, passive uptake cell by permeation across the cell membrane may be so limited as to effectively block drug uptake into the cells.

Several methods for enhancing drug uptake into cells have been proposed. In one general approach, a drug is administered in modified or prodrug form, *e.g.*, with masked charged, for transport into cells. The drug can then be enzymatically converted to an active form within the cells.

Alternatively, the drug compound may be coupled to a carrier molecule, such as transfectin, for transport across the cell membrane. Once inside the cell, the carrier moiety may be removed enzymatically, *e.g.*, by an intracellular esterase or protease.

Another approach to enhancing drug uptake by cells exploits the ability of many cells to engulf particles by endocytosis. Here the drug compound is entrapped in particles, typically particles with sizes less than 200-300 μm, with the particles being administered for targeting to the cells of interest. Liposomes and polymer microparticles are examples of carrier particles that have been used for this purpose.

This approach is limited to certain cell types only, e.g., macrophages, which are active in particle uptake. Another limitation of the approach is that the normal course of intracellular processing involves particle

uptake into lysosomes, where the therapeutic compound, *e.g.*, a nucleic acid, can be enzymatically degraded.

Still another approach to enhancing drug uptake by cells involves the use of fusogenic particles designed to fuse with a target cell membrane, releasing the particle contents into the cell interior. Inactivated virus particles have been widely proposed for this purpose, particularly in the context of gene therapy, for introducing large nucleic acid strands into cells. Virus-like particles composed of fusion-promoting viral proteins embedded in artificial lipid bilayer membranes embedded are another example. In both cases, safety concerns and the expense associated with growing, isolating, and deactivating viral components may limit this approach.

It would therefore be desirable to provide a drug-delivery vehicle that substantially overcomes problems associated with present methods of drug delivery to cells, as outlined above.

4. Summary of the Invention

In one aspect, the invention includes a vesicle composition for use in delivering a therapeutic agent to target biological cells. The composition includes artificial lipid vesicles (i) composed of vesicles-forming lipids that include at least 10 mole percent of a plasmalogen glyceryl lipid having a small-volume polar head group, *e.g.*, phosphatidic acid, phosphatidyl ethanolamine, and phosphatidyl serine, and (ii) that contain the therapeutic agent in entrapped form. An isolated fusion protein in the composition is effective to facilitate fusion of the lipid vesicles with the cells when the cells, vesicles, and protein are brought together.

The fusion protein may be present in solute form in a suspension of the lipid vesicles, or may be attached to the vesicle surfaces, e.g, by covalent coupling to

hydrophilic polymer chains attached to the vesicles, or via a hydrophobic moiety in the protein which anchors the protein to the vesicle membrane.

Where the composition is used for intravenous administration, the vesicles preferably have sizes in the 30-80 nm range, for extended blood circulation lifetime. The vesicles may further include cell-specific targeting molecules carried on the vesicle surfaces for binding the vesicles specifically to target cells.

One preferred composition of vesicle-forming lipids includes 10-80 mole percent plasmalogen phospholipid, 3-15 percent mole negatively charged phospholipid, 10-60 mole percent cholesterol, and 0-40 mole percent neutral phospholipid; preferably 20-40 mole percent plasmenyl ethanolamine, 3-10 mole percent phosphatidylserine, 30-50 mole percent cholesterol, and 20-40 mole percent phosphatidylcholine.

One exemplary fusion protein is an isoform of glyceraldehyde-3-phosphate dehydrogenase. The protein, and its use in promoting membrane fusion, also form part of the invention. Viral fusion proteins represent another class of suitable fusion proteins. Another fusogenic protein useful in the invention is an N-ethylmaleimide (NEM)-sensitive fusogenic protein.

A variety of therapeutic compounds, including nucleic acid strands and nucleic acid analogs, *e.g.*, useful as anti-sense agents, may be delivered. In one embodiment, the therapeutic agent is a membrane protein or glycoprotein effective to enhance a selected activity of target cells, when incorporated into target-cell membranes, where the therapeutic agent is present in the vesicle membranes which are to be fused with the target cells.

The composition is useful in a method of delivering a therapeutic agent to target cells, by bringing the cells,

lipid vesicles, and fusion protein together under conditions suitable for vesicle fusion with the cells.

In a more general aspect, the invention includes a drug-delivery vesicle composition comprising artificial lipid vesicles (i) composed of vesicles-forming lipids that include at least 10 mole percent, and preferably 20-50 mole percent, plasmalogen glyceryl lipid with a relatively small-volume polar head group, and (ii) containing the therapeutic agent in entrapped form.

In one embodiment, the vesicle-forming lipids include 30-70 mole percent plasmenyl ethanolamine and 30-70 mole percent phosphatidylcholine. In another embodiment, the vesicles are composed of between 20-40 mole percent plasmenyl phospholipid with a small volume polar head group, 3-10 mole percent phosphatidylserine, 30-50 mole percent cholesterol, and 20-40 mole percent phosphatidylcholine.

The latter embodiment may further include an isolated fusion protein effective to facilitate fusion of the lipid vesicles with target cells, when the cells, vesicles and protein are brought together.

In still another aspect, the invention provides components for forming a lipid vesicle composition containing first and second reagents capable of interacting with one another. The components include first and second populations of artificial lipid vesicles containing the first and second reagents, respectively. The first population of vesicles is composed of vesicle-forming lipids containing at least 10 mole percent of a plasmalogen phospholipid, and the second population of vesicles is composed of vesicle-forming lipids effective to allow fusion with the first-population vesicles, when the two populations are brought into contact with one another.

The components may further include a fusion protein effective to promote fusion between vesicles in the two different populations.

Plasmenyl phospholipids useful in the invention may be prepared, in accordance with another aspect of the invention, by subjecting at least one ethanolamine glycerophospholipid to an alkaline methanolysis reaction to produce a methanolysis reaction product. The methanolysis reaction product is separated into fractions and a fraction or fractions comprising at least one lysoplasmenyl phospholipids, *e.g.*, plasmenyl ethanolamine, and the head group of the lysoplasmenyl phospholipids, *e.g.*, ethanolamine, is protected with a chemical protection compound. The protected lysoplasmenyl ethanolamine is modified with acyl chloride to give a modified product including at least one protected plasmalogen based phospholipid. Finally, the protected plasmalogen based phospholipid is reacted with a neutral deprotecting agent to give at least one plasmalogen based phospholipid.

These and other objects and features of the invention will be more fully appreciated when the following detailed description of the invention is read in conjunction with the accompanying drawings.

Description of the Drawings

Fig. 1 is a plot of calcium induced fusion of phosphatidylserine lipid vesicles with lipid vesicles comprised of equimolar mixtures of phosphatidylcholine, (PC), and ethanolamine glycerophospholipids. The fluorescence profiles represent the fusion of 16:0-18:1 phosphatidylserine (PS) SUVs with vesicles containing equimolar mixtures of POPC/18:0-20:4 plasmenyl ethanolamine (A), POPC/16:0-18:1 plasmenyl ethanolamine (B), POPC/18:0-20:4 phosphatidyl ethanolamine, (PE), (C), and POPC/16:0-18:1 phosphatidyl ethanolamine (D).

Fluorescence tracings from four independent preparations, all performed in triplicate, were averaged after normalization to the maximum fluorescence increase which would occur after all vesicles fused. Fig. 2 is a plot of the comparison of the effect of endogenous or plasmalogen-depleted bovine brain ethanolamine glycerophospholipids on calcium-induced fusion of phosphatidylserine lipid vesicles with phosphatidylcholine/ethanolamine glycerophospholipid (1:1) lipid vesicles. Fluorescence profiles represent the fusion of 16:0-18:1 phosphatidylserine SUVs with vesicles containing equimolar mixtures of POPC/bovine brain PE (A), or POPC/plasmalogen-depleted bovine brain PE (B). Fluorescence tracings from four independent preparations, all performed in triplicate, were averaged after normalization.

Fig. 3 is a plot of N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)-1,2-dihexadecanoyl-*sn*-glycerol-3-phosphoethanolamine/N-(lissamine rhodamine B sulfonyl)-1,2-dihexadecanoyl-*sn*-glycero-3-phosphoethanolamine (NBD-PE/Rh-PE) fusion assay of calcium-induced fusion of phosphatidylserine lipid vesicles with lipid vesicles comprised of equimolar mixtures of phosphatidylcholine and ethanolamine glycerophospholipids. Fluorescence profiles represent the fusion of 16:0-18:1 phosphatidylserine SUVs with vesicles containing equimolar mixtures of POPC/16:0-18:1 plasmenyl ethanolamine (A), POPC/bovine brain PE (B), and POPC/16:0-18:1 phosphatidylethanolamine (C). Fluorescence tracings were obtained from two independent preparations, performed in quadruplicate, which were averaged after normalization.

Fig. 4 is a plot of terbium/dipicolinic acid (Tb/DPA) assay of calcium induced fusion of phosphatidylserine lipid vesicles with lipid vesicles comprised of equimolar mixtures of phosphatidylcholine and ethanolamine glycerophospholipids. Fluorescence profiles represent the

fusion between 16:0-18:1 phosphatidylserine SUVs and vesicles containing equimolar mixtures of POPC/16:0-18:1 plasmenyl ethanolamine (A) or POPC/16:0-18:1-phosphatidylethanolamine (B). Fluorescence tracings from three independent preparations, all performed in quadruplicate, were averaged after normalization to the maximum fluorescence increase which would occur after all vesicles fused.

Fig. 5 is a plot of calcium-induced fusion of vesicles comprised of equimolar ternary mixtures of serine, choline, and ethanolamine glycerophospholipids. Fluorescence profiles represent the fusion of SUVs comprised of POPS/POPC/16:0-18:1 plasmenyl ethanolamine (1:1:1) (A), POPS/POPC/plasmalogen-depleted bovine brain PE (1:1:1) (B), POPS/POPC/plasmalogen-depleted bovine brain PE (1:1:1) (C), and POPS/POPC/16:0-18:1 phosphatidylethanolamine (1:1:1) (D). Fluorescence tracings were obtained from three independent preparations, performed in quadruplicate, which were averaged after normalization.

Fig. 6 is a plot of calcium-induced fusion of small unilamellar vesicles comprised of physiologically relevant ratios of phospholipids. Fluorescence profiles represent the fusion of SUVs comprised of POPC/16:0-18:1 plasmenyl ethanolamine/POPS (45:45:10) (A), and POPC/16:0-18:1 phosphatidylethanolamine/POPS (45:45:10) (B). Fluorescence tracings were obtained from two independent preparations, performed in quadruplicate, which were averaged after normalization.

Fig. 7 shows anion exchange chromatography of dialyzed rabbit brain cytosol and characterization of the substrate selectivity of the protein catalyzing membrane fusion. Rabbit brain cytosol was prepared and loaded onto a previously equilibrated DE-52 column. The column was developed with a linear NaCl gradient (0-400 Mm NaCl) and column eluents were assayed for their ability to catalyze

membrane fusion utilizing physiologically-modeled small unilamellar vesicles (SUVs) as described in "Materials and Methods". Vesicles were comprised of 16:0-18:1 phosphatidylcholine (27%), 16:0-18:1 phosphatidylserine (6%), cholesterol (40%) and 27% of each of the following ethanolamine glycerophospholipids: 18:0-20:4 plasmenylethanolamine (—■—); 16:0-18:1 plasmenylethanolamine (—□—); 18:0-20:4 phosphatidylethanolamine (—•—); or 16:0-18:1 phosphatidylethanolamine (—○—). (——), uv absorbance at 280 nm; (– –), NaCl gradient.

Fig. 8a shows Mono Q chromatography of eluents from GTP affinity chromatography containing membrane fusion-catalyzing activity. Active fractions from the GTP-agarose column were pooled, diluted tenfold with equilibration buffer, and loaded onto a Mono Q column. The column was developed with a sodium chloride gradient (– –) and aliquots of column eluents were assayed for fusion-catalyzing activity utilizing physiologically-modeled vesicles. The UV absorbing peaks eluting between 13 and 15 ml result from the elution of metabolites, NADH and other non-protein moieties. (—) uv absorbance at 280 nm. Fig. 8b shows an autoradiograph of ^{125}I Boulton-Hunter labeled and SDS-PAGE separated proteins from both the Mono Q column load and column eluents containing membrane fusion-catalyzing activity.

Fig. 9A shows comparisons of glyceraldehyde 3-phosphate dehydrogenase activity and membrane fusion activity in fractions from GTP-agarose and Mono Q chromatographies. Left panel: GAPDH activity was assessed spectrophotometrically by production of NADH. Enzyme aliquots were obtained from either the GTP-agarose affinity column (3 mM GTP eluent (A) or the 20 mM tripolyphosphate/10 mM NAD^+ eluent (B)) or from the concentrated void volume of the Mono Q column (C) (concentrated using an Amicon Microcon-10) or from the

Mono Q eluents catalyzing membrane fusion activity (D) (also concentrated using an Amicon Microcon-10). Fig. 9B shows SDS-PAGE (10-15% acrylamide PhastGel) analysis of aliquots of enzyme fractions utilized in samples A-D visualized by silver staining.

Figs. 10A-10C show ethanolamine glycerophospholipid selectivity of the GAPDH isoform mediating membranes fusion at consecutive steps in its purification, including the DE-52 void volume (0.03 mg/ml) (10A), the active fraction eluting from HiLoad SP Sepharose (10B), and the active fraction from GTP-agarose chromatography (10C).

Fig. 11 shows inhibition of GAPDH isoform-catalyzed membrane fusion by (D)-glyceraldehyde 3-phosphate. Physiologically-modeled SUVs were prepared (concentration = 200 μM). The other chamber contained the Pi_3/NAD^+ eluent from GTP-agarose chromatography (0.004 mg/ml) which was previously incubated with (D)-glyceraldehyde 3-phosphate at the indicated concentrations for 30 seconds at 37°C. The rates of membrane fusion from four separate determinations of two preparations were averaged and expressed as a percentage of maximum fusion.

Fig. 12a shows the concentration dependence of the inhibition of membrane fusion activity by the 155.D2.2 monoclonal antibody, and concentration independence of the inhibition of membrane fusion by anti-actin (A) and anti-GAPDH 155.B5.4 (B) monoclonal antibodies. Fig. 12b shows a demonstration of the specificity of both 155.D2.2 and 155.B5.4 for GAPDH when used to probe a Western blot of rabbit brain cytosol separated on 11% SDS-PAGE. The specificity of monoclonal antibodies was determined by visualizing the distribution of bound primary monoclonal antibody after treatment with [^{125}-I] rabbit anti-mouse IgG (secondary antibody) and subsequent autoradiography.

Figs. 13A and 13B show the inhibition of glyceraldehyde (3)-phosphate dehydrogenase activity (13A), but not membrane fusion activity (13B), by koningic acid.

Fig. 14 shows the selectivity of the GAPDH isoform catalyzing membrane fusion for physiologically-modeled vesicles containing distinct mole fractions of plasmenylethanolamine and phosphatidylethanolamine. Physiologically-modeled small unilamellar vesicles consisting of 16:0-18:1 phosphatidylcholine (27%), ethanolamine glycerophospholipid (27%), 16:0-18:1 phosphatidylserine (6%) and cholesterol (40%) were prepared (final lipid concentration of 200 μM) and placed in one chamber of a stopped-flow apparatus. Aliquots of the HiLoad SP Sepharose column eluent (protein concentration = 0.002 mg/ml) were placed in the other chamber, samples were rapidly mixed in a stopped flow apparatus and membrane fusion was quantified. Ethanolamine glycerophospholipids included 100% plasmenyl ethanolamine (A) 75% plasmenyl ethanolamine and 25% PE (B); 50% plasmenyl ethanolamine and 50% PE (C); and 25% plasmenyl ethanolamine and 75% PE (D;

Fig. 15 shows the selectivity of the GAPDH isoform catalyzing membrane fusion for physiologically-modeled vesicles containing increasing amounts of PS. Vesicles were composed of 16:0-18:1 PC/18:0-20:4 plasmenyl ethanolamine/cholesterol (27/27/40 mole percent) and varying mole percentages of phosphatidylserine (6% phosphatidylserine (A), 12% phosphatidylserine (B), 3% phosphatidylserine (C) or 0% phosphatidylserine (D)).

Fig. 16 shows the dependence of GAPDH isoform-catalyzed membrane fusion on the cholesterol content of vesicles. Physiologically-modeled SUVs containing phosphatidylcholine/18:0-20:4 plasmenylethanolamine/phosphatidylserine (45/45/10 mole ratio) and selected mole percentages of cholesterol (*i.e.*, 40% cholesterol (A), 20% cholesterol (B), or 0% cholesterol (C)) were placed in one chamber of a stopped-flow apparatus. The other chamber contained aliquots of the active fraction from GTP-agarose chromatography (*i.e.*,

20 mM tripolyphosphate/10 mM NAD^+ (Pi_3/NAD^+)), at a protein concentration of 0.004 mg/ml. Samples were rapidly mixed and membrane fusion was quantified. Results represent the average of six recordings from two independent preparations expressed as a percentage of maximal fusion.

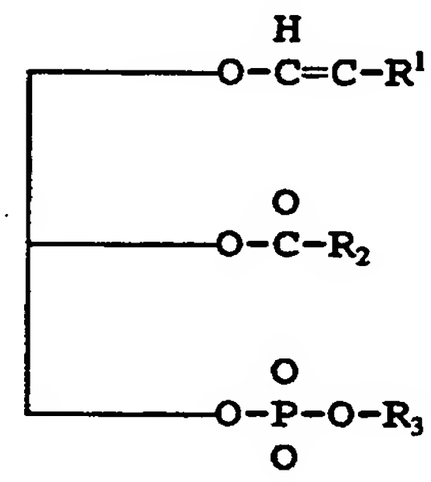
Detailed Description of the Invention

I. Definitions

Unless otherwise indicated, the terms below have the following definitions herein:

"Artificial lipid vesicles" refers to lipid bilayer vesicle or lipid vesicles prepared from isolated vesicle-forming lipids, typically having a known composition of lipid components.

"Plasmalogen phospholipid" refers to a vesicle-forming lipid of the form:



where; R_1 and R_2 are hydrocarbon chains having lengths from about 8-24 atoms, and which may include unsaturated carbon-carbon bonds, and R_3 is a phosphate-attached polar head group, *e.g.*, serine, ethanolamine, choline, or inositol, or a proton (phosphatidic acid) head group.

"Plasmenyl phospholipid with a small-volume polar head group" refers to a plasmenyl phospholipid in which R_3 is serine, ethanolamine, of small-volume analogs thereof, *e.g.*, cycloserine, monomethylethanolamine or other head groups whose volume is substantially less than that of choline.

"Plasmenyl ethanolamine" or "plasmenyl ethanolamine phospholipid" or "plasmenyl ethanolamine glycerophospholipid" refers to a plasmalogen phospholipid of the type shown above, where R_3 is ethanolamine.

"Plasmenyl glyceryl lipid with a small-volume polar head group" includes, in addition to plasmenyl phospholipids with a small-volume polar head group, diacyl glycerol or analog thereof in which one of the hydrocarbon chains in the molecule is linked to the glycerol backbone through a vinyl ether linkage (alkenyl acylglycerol or analog with a small-volume, non-phosphate containing head group).

II. Plasmenyl Phospholipids and Vesicle Fusion

According to one aspect of the invention, it has been discovered that the ability of artificial lipid vesicles to fuse with target cell membranes can be enhanced significantly by addition to the vesicles of plasmenyl glycerol lipid with a small-volume polar head group. This section describes methods of preparing plasmenyl phospholipids to this type, and the fusion properties of lipid vesicles containing such lipids.

A. Preparation of Plasmenyl Phospholipids

Example 1 describes a method for producing a synthetic plasmenyl phospholipid for use in the invention. The method includes the steps of first preparing a plasmenyl phospholipid-containing lipid mixture from a natural source, *e.g.*, brain tissue. The lipid mixture is subjected to alkaline methanolysis to produce a methanolysis reaction products, which are separated into fractions. Fraction(s) containing at least one lysoplasmenyl phospholipid, *e.g.*, plasmenyl ethanolamine, are recovered and the head group, *e.g.*, ethanolamine, of the lysoplasmenyl lipid is protected with a chemical protection compound to give a protected lysoplasmenyl

lipid. The protected lysoplasmenyl lipid is modified with acyl chloride to give a modified product including at least one protected plasmalogen based phospholipid. Finally, the protected plasmalogen based phospholipid is reacted with a neutral deprotecting agent to give at least one plasmalogen based phospholipid. This method is described in Glaser, 1994. Other methods of isolating and/or synthetically preparing plasmenyl phospholipids are known.

B. Membrane Fusion Assays

Fusion assays employed to demonstrate vesicle fusion are detailed in Example 2. Briefly, lipid vesicles whose fusion properties are to be tested are mixed with small unilamellar vesicles (SUVs) formed entirely of phosphatidyl serine (PS), which present a highly fusogenic "target". The vesicles to be tested typically are formed by sonication down to SUV sizes, *e.g.*, in the 30-80 nm size range.

Several methods are available for measuring membrane fusion. In the octadecyl (R_{11}) fusion assay described in Example 2A, PS SUVs prepared with 4% octadecylrhodamine (R18) are mixed with unlabeled test vesicles, and membrane fusion is followed by the temporal dequenching of R_{11} observed at 590 nm after excitation at 560 nm.

In the NBD-PE/Rh-PE assay detailed in Example 2B, NBD-PE (N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)-1,2-dihexadecanoyl-*sn*-glycerol-3-phosphoethanolamine), and Rh-PE are incorporated into PS SUV's and the vesicles are mixed with test vesicles. Vesicle fusion is followed by the change in fluorescence emission at 530 nm, after emission at 464 nm.

Vesicle contents mixing assays are performed by incorporating DPA (dipicolinic acid) probe into PS SUVs and $Tb(Cl)_3$ into test vesicles. Vesicle fusion is

monitored through the formation of the fluorescent Tb/DPA complex measured at > 470 nm after excitation at 276 nm.

C. Vesicle Fusion

The effect of plasmenyl phospholipids and phospholipid acyl chain lengths on rates of vesicle fusion, in SUVs composed of equimolar amounts of PC and plasmenyl PE or PE was investigated. As seen in Fig. 1, plasmenyl ethanolamine lipids vesicles (plots A and B) gave substantially higher rates of fusion than corresponding vesicle formulations containing the PE (plots C and D, respectively), and longer acyl chain lengths (18:0, 20:4) in both plasmenyl and PE phospholipids (plots A and C) gave substantially higher fusion rates than phospholipids with shorter acyl chain lengths (16:0, 18:1).

It should be noted that vesicles containing plasmenyl ethanolamine did not induce alterations in the calcium profile or threshold of vesicle fusion. Both semisynthetic 18:0, 20:4 plasmenyl ethanolamine and 18:0, 20:4 plasmenyl ethanolamine purified from canine myocardium gave identical fusion results. Additionally, measured fusion rates of PC/PE vesicles containing the R_{18} label to labeled PS SUVs were identical to those obtained in systems containing the R_{18} label in the PS SUVs.

Fig. 2 shows a comparison of control and plasmalogen-depleted bovine brain ethanolamine glycerophospholipids in a PC/PE and PS vesicle fusion system. The results demonstrate that vesicles containing native bovine brain ethanolamine glycerophospholipids (plot A) were twice as fusogenic as those containing plasmalogen-depleted ethanolamine glycerophospholipids (plot B). Bovine brain PE, which contains a mixture of both plasmalogen and diacyl-PE, supports a fusion rate that falls between that of mixed vesicles containing only plasmalogen PE and those containing only diacyl-PE as shown by comparing Figs. 1

and 2. Plasmalogen-depleted bovine brain PE supported vesicle fusion rate similar to those manifested by phosphatidylethanolamine containing oleic acid at the *sn*-2 position.

Similar results were obtained in the NBD-PE fusion assay. Vesicles composed of equal molar mixtures of PC and 16:0-18:1 plasmenyl ethanolamine fused to PS SUVs three times more rapidly fused than vesicles comprised of equal molar mixtures of PC and 16:0-18:1 phosphatidylethanolamine, as determined by the dequenching of NBD-PE fluorescence by Rh-PE as a surface area increased after vesicle fusion. Although detailed full time course kinetic analysis of vesicle fusion processes demonstrates substantial complexity, the results herein largely reflect differences in the initial rates of membrane fusion where contribution from second order processes are minimized.

Similar results were also obtained in a contents mixing assay, as seen in Fig. 4. The fusion of vesicles composed of PS with vesicles of equimolar mixtures of PC/PE for both 16:0-18:1 plasmenyl ethanolamine and 16:0-18:1 phosphatidyl ethanolamine exhibit a time course, initial rate, and extent similar to that observed in the R_{18} fusion assay (compare Fig. 4 to Fig. 1). The positive correlation of rate constants derived from contents mixing and lipid mixing fusion assay shows that the lipid-mixing assays reflect, in large part, *bona fide* membrane fusion and do not reflect vesicle apposition and lipid transfer.

It is known that lipid vesicles having a smaller diameter and higher radius of curvature, and consequently more internal strain, exhibit faster fusion rates in general than those having a larger diameter, a lower radius of curvature and less internal strain. To verify that observed differences in fusion rates were attributable to properties of the vinyl ether linkage and did not result from differences in vesicle size, the

diameter of diacyl-plasmalogen-containing vesicles using [^{14}C] inulin was compared. There were no significant differences in size between the various binary mixtures of PC and PE SUVs comprised of distinct ethanolamine glycerophospholipid subclasses as seen in Table I. The diameter of PS vesicles were approximately 1.2 times larger than SUVs comprised of equal molar mixtures of choline and ethanolamine glycerophospholipids.

Table I

Vesicle Incorporation of [^{14}C]inulin

Incorporated/μmole Liposome Composition	[^{14}C]inulin of Lipid (DPN)
Phosphatidylserine POPC/16:0-18:1	72,500 ± 4,600 43,600 ± 3,700
Plasmenylethanolamine POPC/16:0-18:1	43,200 ± 3,200
Phosphatidylethanolamine POPC/Bovine Brain PE	40,900 ± 3,000

The effect of plasmenyl ethanolamine lipids on lipid vesicle fusion in vesicles containing in more complex lipid mixtures containing PS, PC, and plasmenyl lipids was also examined with the R_{11} fusion assay. The assay results are found in Fig. 5.

The presence of plasmenyl ethanolamines induces an even greater increase in relative membrane fusion rates in this liposome fusion system. Vesicles containing 16:1-18:1 plasmenyl ethanolamine were six times more fusogenic than those containing 16:0-18:1 phosphatidylethanolamine. Similarly, vesicles containing bovine brain PE were more than twice as fusogenic as those containing plasmalogen-depleted PE in this system. To approach even more closely the physiologic complement of phospholipid classes in a synaptic vesicle, lipid vesicles comprised of 45% PC/45% PE/10% PS were also assayed and the results are found in

Fig. 6. Although fusion rates were significantly slower in this system in comparison to the previous systems, vesicles containing 45% 16:0-18:1 plasmenyl ethanolamine demonstrate an initial rate of membrane fusion significantly greater than vesicles containing corresponding amounts of 16:0-18:1 phosphatidylethanolamine.

The results of these experiments demonstrate that the covalent nature of the *sn*-1 aliphatic constituents in glycerophospholipids is an important determinant of the rate of membrane fusion. More generally, enhanced vesicle fusion is observed in lipid vesicles containing at least 10 mole percent, and preferably 30-70 mole percent of a plasmenyl phospholipid containing a small-volume head group. These results are substantiated through fast-flow kinetic analysis using multiple independent methods to quantify membrane fusion, including both lipid-mixing and internal contents mixing fusion assays.

D. <u>Plasmenyl-Vesicle Composition</u>

In one aspect, the invention includes a lipid vesicle composition which exploits the greater fusogenic activity of lipid vesicles containing plasmalogen glyceryl lipids with small-volume polar head groups. In particular, the vesicles containing such lipid, in an amount of at least 10 mole percent, are used for delivery of a therapeutic agent entrapped in the vesicles for delivery to a target cell.

The entrapped therapeutic agent may be any of a large number of agents that can be entrapped in lipid vesicles, including water-soluble agents that can be stably encapsulated in the aqueous compartment of the vesicles, lipophilic compounds that stably partition in the lipid phase of the vesicles cells, or agents that can be stably attached, *e.g.*, by electrostatic attachment to the outer vesicle surfaces. Exemplary water-soluble compounds

include small, water-soluble organic compounds, peptides, proteins, oligonucleotides and gene fragments.

Lipid vesicles containing the plasmalogen lipids and an entrapped agent are prepared according to well-known methods, such as hydration or a lipid film, reverse-phase evaporation, and solvent infusion. Other lipid vesicles components used in the preparation of the vesicles include conventional phospholipids, such as PC, PS, and/or PE, and may also include cholesterol or cholesterol analogs. One preferred composition, disclosed in Example 3, includes equimolar amounts of PC and plasmenyl PE.

Another preferred lipid composition, particularly for use in a lipid composition in combination with a fusion protein (Section 3 below) includes 20-40 mole percent plasmenyl phospholipid, 3-10 mole percent phosphatidylserine, 30-50 mole percent cholesterol, and 20-40 mole percent PC.

Where the composition is used for intravenous administration, the vesicles are preferably SUVs, *i.e.*, in the size range 30-80 nm, for longer circulation time. Methods for producing sized liposomes, *e.g.*, by sonication or extrusion through defined pore-size membranes are well known.

The vesicles may be further designed, *e.g.*, by the addition of surface receptors or ligands, for cell-specific binding to target cells.

The vesicles are employed, in one general application, for delivery of a therapeutic agent to target cells, via vesicle fusion with the target cell plasma membrane. A biologically active agent can also be disposed within a bilayer of a vesicle and transferred to a cell using the compounds of this invention whereby the compound nor compounds, disposed within the lipid bilayer, becomes integrated within the cell membrane.

In another general application, the vesicle composition is used as an anti-viral agent, acting as a

target-cell decoy for virus circulating in the bloodstream. This application relies on the ability of a virus fusion protein to catalyze the fusion to plasmalogen containing bilayer membranes, discussed in the section below.

III. Protein-Mediated Vesicle Fusion

In accordance with another aspect of the invention, it has been discovered that plasmalogen-containing lipid vesicles are rapidly fused with bilayer membranes in the presence of fusion proteins, including a newly discovered fusion protein that has been identified as an isoform of glyceraldehyde-3-phosphate dehydrogenase (GAPDH).

A. Isolation and Characterization of GAPDH Isoform

The isolation of a GAPDH isoform that has been discovered to catalyze vesicle fusion, in accordance with the invention, is described in Example 5. Briefly, a mouse-brain homogenate was centrifuged at high speed to produce a cytosol. Dialyzed cytosol was fractionated on a DE-52 column, and the fractions were assayed in a vesicle fusion assay. The lipid vesicles that were used in the assay were composed of 27 mole percent of a selected PC lipid, one composition with plasmalogen ethanolamine, 6 mole percent PS, 40 mole percent cholesterol, and 27 mole percent PC. These vesicles show little measurable self-fusion activity, as evidenced by change in fluorescence in an R_{11} assay (half of the vesicles labeled with R_{11}). The cytosol itself showed no measurable ability to catalyze the fusion of the vesicles, either for plasmalogen or PE containing vesicles. However, the void volume from the column showed high vesicle fusion activity. Surprisingly, this activity was confined to vesicles containing plasmalogen phospholipids (Fig. 7). The reasons underlying the cryptic nature of this activity in crude cytosol have been elaborated, showing that the cytosol

contains a potent endogenous protein inhibitor of fusion activity.

Fractions from the void volume containing fusion-catalyzing activity were pooled, filtered and loaded onto a HiLoad SP Sepharose column. After extensive washing, proteins with membrane fusion activity were eluted utilizing a nonlinear sodium chloride gradient from 0 to 0.5 M NaCl. Active fractions were pooled, and loaded onto a GTP-agarose column. After extensive washing, fractions were eluted with 5 mM GMP, 3 mM GTP, and finally in buffer alone. Membrane fusion activity was eluted with 20 mM tetrasodium tripolyphosphate and 10 mM NAD^+.

GTP-agarose affinity eluents were further purified either by reverse phase HPLC (in preparation for protein sequencing) or by Mono Q anion exchange chromatography. Active fractions were pooled, and loaded onto a Mono Q PC 1.6/5 column previously equilibrate buffer C at a flow rate of 200 μl/minute. Fusion-catalyzing activity was eluted using a continuous gradient from 0 to 500 mM NaCl. The fraction associated with fusion activity is shown in Fig. 8. At this stage, a final preparation was obtained which was 790-fold purified. A substantially pure protein band was obtained on SDS PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis). The purified protein migrated with a molecular weight of 38 kDal.

The membrane fusion protein, purified as described above, was applied to a Vydac Reverse Phase HPLC column. A single homogeneous 38 kDa band was obtained and submitted for N-terminal Edman degradation sequencing. Twenty-three amino acids of N-terminal sequence were obtained and found to be homologous to GAPDH.

Table II

Sequence of the Membrane Fusion Protein and Homology with GAPDH

VKVGVNGFGRIGRLVTRAAFNSG	Membrane Fusion Protein
VKVGVNGFGRIGRLVTRAAFNSG	Human GAPDH (muscle)
VKVGVNGFGRIGRLVTRAAFNSG	Human GAPDH (liver)
VKVGVNGFGRIGRLVTRAAFNSG	Bovine GAPDH
1 5 10 15 20 aa	

The following evidence demonstrated that the isolated fusion protein is an isoform of GAPDH:

1. Western blots of the purification fractions from above, including the final purification material, all showed a single 38 kDal when probed with anti-GAPDH antibody (prepared as in Example 5).

2. The N-terminal sequence of the isoform is identical with that of several GAPDH enzymes from several sources (Table II above).

3. Membrane fusion activity was inhibited by D-glyceraldehyde 3-phosphate with a K_i similar to the association constant of glyceraldehyde 3-phosphate with GAPDH (Fig. 11).

4. A monoclonal antibody directed against GAPDH (*i.e.*, antibody 155.D2.2) specifically inhibited membrane fusion in a dose-dependent manner, while other monoclonal antibodies (*e.g.*, either other antibodies directed against GAPDH (*e.g.*, 1.55.B5.4) or those directed against other non-relevant proteins (*e.g.*, actin)) did not inhibit membrane fusion activity even at concentrations of monoclonal antibody which were 10-fold higher than those which inhibited fusion activity (Fig. 12).

5. Koningic acid, a known inhibitor of GAPDH enzyme activity (Sakai, *et al.*, 1991) which covalently binds to the active site cysteine (cysteine 149) failed to inhibit

membrane fusion activity at concentrations which maximally inhibited GAPDH enzyme activity (Figs. 13A and 13B).

Further characterization of the membrane fusion-catalyzing activity in the void volume demonstrated that it was trypsin-sensitive, calcium-independent, neutral-active (maximal activity was present between pH 6 and 7), heat-labile (activity was ablated by heating for 3 minutes at 90°C), and inactivated by DTNB (1 mM for 30 minutes at 37°C) but was not inhibited by N-ethylmaleimide (1 mM for 60 minutes at 37°C) (data not shown). Furthermore, membrane fusion activity catalyzed by void volume eluents was not due to the fusion of labeled R_{18} vesicles with endogenous lipid carried through in the void volume since dequenching of R18 fluorescence did not occur in the absence of added acceptor vesicles.

Examination of the tissue specificity of the fusion activity revealed appreciable levels only in brain and muscle (rabbit hind leg muscle) with only diminutive amounts of activity present in liver and kidney (100-fold less than that manifest in brain). Finally, a crude surgical separation of rabbit brain white matter from gray matter demonstrated that fusion activity in void volume eluents derived from gray matter was threefold higher than those derived from white matter (data not shown). Thus, the void volume from anion exchange chromatography of rabbit brain cytosol contains a tissue-specific protein that catalyzes the calcium-independent fusion of membrane bilayers containing plasmenylethanolamine but not phosphatidylethanolamine.

B. <u>Lipid Vesicle Composition and Fusion Properties</u>

Studies were carried out in support of the invention to determine optimal lipid composition for protein-catalyzed vesicle fusion. First, the ethanolamine glycerophospholipid subclass requirements of the fusion protein at each stage of the purification procedure were

examined. Membrane fusion activity was highly selective for vesicles containing plasmenylethanolamine (compared with phosphatidylethanolamine) at each stage of the purification procedure (Figs. 10A-10C). Furthermore, a 2- to 4-fold selectivity for plasmalogens containing arachidonic acid in comparison to oleic acid at the *sn-2* position (synaptic membranes are substantially enriched in arachidonic acid-containing molecular species) was observed.

To quantify the dependence of membrane fusion activity on the fractional percent of plasmenylethanolamine present in the vesicles undergoing membrane fusion, the mole fraction of ethanolamine glycerophospholipids represented by plasmenyl ethanolamine was varied from 0-100%. Membrane fusion activity increased as the mole fraction of plasmenylethanolamine in the vesicles was increased up to 75% plasmenylethanolamine with no additional increment at higher plasmenylethanolamine mole fractions (Fig. 14).

To determine the importance of serine glycerophospholipids and cholesterol in facilitating fusion mediated by the purified protein, fusion rates were quantified in vesicles containing selected concentrations of phosphatidylserine (0-12 mole %) and cholesterol (0-40 mole %). In vesicles in which the serine glycerophospholipid content was varied from 0-12%, the most rapid rates of membrane fusion were present in vesicles containing 6 mole% PS (Fig. 15) (note that this approximates the percentage of PS found in synaptosomal membranes (Westhead, 1987; Cullis and Hope, 1991). Importantly, vesicles containing only 3% PS or less could not be induced to fuse by the membrane fusion protein under the conditions employed. Vesicles containing 12 mole% PS fused at rates that were considerably less than those manifest at 6 mole % PS, demonstrating that neither bulk alterations in membrane surface charge nor

alterations in physical properties of the vesicle is the sole mechanism through which changes in PS content facilitate protein-mediated membrane fusion (in contrast to non-protein-mediated Ca^{2+}-dependent vesicle fusion).

Next, the concentration of cholesterol in the vesicles was varied from 0-40 mole%. The membrane fusion protein possessed an obligatory requirement for cholesterol, with little or no fusion manifest in vesicles lacking cholesterol (Fig. 16). Protein-facilitated membrane fusion activity induced by cholesterol increased in a dose dependent fashion with the most rapid rates of membrane fusion manifest in vesicles containing a physiologic complement of cholesterol (*i.e.*, 40 mole%). Collectively, these results demonstrate that the membrane fusion activity has an obligatory requirement for physiologic complements of plasmenyl phospholipid with small-volume polar head group-- in this case, ethanolamine-- a negatively charged phospholipid, *e.g.*, PS, and cholesterol, and will not rapidly catalyze membrane fusion in the absence of any one of these constituent lipids of synaptic vesicles and the plasma membrane.

The kinetics of GAPDH isoform-catalyzed membrane fusion was calculated from the high-resolution stopped-flow fusion measurements made above. Calculations of fusion rates were based on the protein concentration of 4 μg/ml and vesicle concentration (800 μm lipid, utilizing the assumptions that ≈ 1000 molecules of lipid are in each vesicle and that the active form of GAPDH is a tetramer (similar to the active form of the dehydrogenase). Utilizing these assumptions, the GAPDH isoform catalyzed the fusion of one pair of vesicles in 1 ms (on average). Since many rounds of vesicle fusion are catalyzed during each assay, it is evident that the fusion protein represents a true catalyst of vesicle fusion and does not mediate the fusion of vesicles in a stoichiometric fashion

(*i.e.*, hundreds of vesicle pairs undergo fusion mediated by one GAPDH tetramer during each assay).

IV. Fusion-Vesicle Composition and Method

This section describes a vesicle composition for use in delivering a therapeutic agent to target biological cells, in accordance with the invention. The composition includes artificial lipid vesicles composed of vesicles-forming lipids that include at least 10 mole percent of a plasmalogen glyceryl lipid with a small-volume polar head group, and an isolated fusion protein effective to facilitate fusion of the lipid vesicles with the cells, when the cells, vesicles and protein are brought together. The therapeutic agent is entrapped in the vesicles.

A. Lipid Vesicles

The lipid vesicles in the composition are formed as above by standard vesicle-forming methods. The lipids employed in the vesicles include at least 10 mole percent of a plasmenyl phospholipid with a small-volume polar head group, *e.g.*, ethanolamine, serine, or phosphatidic acid. Alternatively, the plasmenyl lipid may be diacyl glycerol analog, where one of the hydrocarbon chains is linked to the glycerol backbone through a vinyl ether linkage. The amount of the plasmenyl glycerol lipid is preferably between about between 20-70 mole percent, more preferably 20-40 mole percent.

In accordance with the studies discussed above, the lipid mixture also include 3-15 mole percent of a negatively charged phospholipid, *e.g.*, PS or PA, preferably about 3-10 mole percent PS. 3 below) includes 20-40 mole percent plasmenyl phospholipid, 3-10 mole percent phosphatidylserine, 30-50 mole percent cholesterol, and 20-40 mole percent PC. It will be appreciated that the negatively charged phospholipid may

be contributed by the plasmenyl phospholipid itself, *e.g.*, by plasmenyl PS.

The vesicles also contain cholesterol or a cholesterol analog, *e.g.*, ergosterol, in an amount preferably between 20-50 mole percent cholesterol, more preferably and 30-40 mole percent. The balance of the lipids, if any, is preferably made of PC, in an amount between 0-40 mole percent, preferably 20-40 mole percent. Other vesicle-forming lipids, or lipids that are stably anchored in a lipid bilayer, such as alpha-tocopherol, may be included.

Lipid vesicles containing the plasmalogen lipids and an entrapped agent are prepared according to well-known methods, as described above. Typically, vesicles are formed by hydration of a lipid film, where the compound to be delivered is either included in the lipid film, in the case of a lipophilic compound, or is included in the hydration medium, in the case of a water-soluble therapeutic agent. Alternatively, the therapeutic agent may be loaded into preformed vesicles, *e.g.*, by loading an ionizable compound against a pH gradient.

Where the composition is used for intravenous administration, the vesicles are preferably SUVs, *i.e.*, in the size range 30-80 nm, for longer circulation time. Methods for producing sized liposomes, *e.g.*, by sonication or extrusion through defined pore-size membranes are well known. The vesicles may be further designed, *e.g.*, by the addition of surface receptors or ligands, for cell-specific binding to target cells.

B. <u>Fusion Protein</u>

The fusion protein in the composition is a protein effective to catalyze the fusion of artificial lipid vesicles to target biological cells, *i.e.*, mammalian cell membranes. More particularly, the fusion proteins in the invention are characterized by the ability to catalyze

rapid fusion of lipid vesicles having the lipid composition described above, *e.g.*, 20-40 mole percent plasmenyl ethanolamine, 3-10 more percent PS, 20-40 mole percent cholesterol and 20-40 percent PC. As demonstrated in the studies above, protein-catalyzed fusion occurs to a substantial extent within a few seconds of mixing vesicles and protein, whereas no measurable fusion is observed in the absence of the protein.

One preferred protein in the composition is the GAPDH isoform described in Section III. Additionally, a fusion protein which has been described in relation to vesicle-mediated transport in both mammalian and yeast cells is the so-called NEM (N-ethylmaleimide)-sensitive fusion protein (NSF) and related fusion protein SEC18 (Wilson). Further, peptide fragments of these proteins which retain fusion active are also considered herein to be fusion proteins.

A large number of viral fusion proteins that catalyze viral fusion reactions either under acidic or pH independent conditions have also been described (White, Blobel, Edwardson, Epand, Fredericksen, Ito, Morrison, and Rapaport). Many of these proteins have hemagglutinin activity (White, Tsurodome). All viral proteins studies to date are class I integral proteins having external N termini and internal C termini with a membrane spanning anchoring region. A large number of viral proteins have been cloned and can be prepared by standard recombinant techniques (White).

Because the fusion protein is capable of promoting the fusion of vesicles in suspension, it must either be maintained separate from the vesicles, prior to use, or maintained in the presence of an inhibitor of the protein's vesicle-fusion activity. Thus, for example, when the vesicles are administered intravenously for vesicle fusion at a target site, the fusion protein may be administered as a separate agent, *e.g.*, in solution or

carried on non-fusogenic lipid vesicles, to provide therapeutic vesicles and fusion protein at the *in vivo* target site.

Alternatively, the fusion protein may be present in a suspension of the therapeutic vesicles in a composition that also contains a soluble inhibitor of protein-catalyzed vesicles fusion. For example, where the fusion protein is GAPDH isoform, high concentrations of glutaraldehyde-3-phosphate or high concentration of a low-affinity anti-GAPDH antibody may be employed (see Figs. 11 and 12B). One inhibitor of GAPDH isoform fusion which has been discovered herein is a 55 KDal tubulin protein isolated from brain cytosol, prepared according to the isolation procedures detailed in Example 6. In studies performed in support of the invention, it was found that:

1. tubulin-Sepharose affinity columns specifically bind GAPDH isoform;

2. tubulin-mediated inhibition of GAPDH isoform catalyzed membrane fusion was not attenuated by large increases in vesicle concentration;

3. tubulin-mediated inhibition of membrane fusion was stoichiometric with GAPDH; and

4. tubulin-mediated inhibition of GAPDH-catalyzed membrane fusion was dependent upon the residence time of tubulin with GAPDH isoform, and independent of contact time of tubulin with vesicles.

In the case of NEM-sensitive fusion protein, the inhibitor of membrane fusion may be a suitable reducing agent, *e.g.*, N-ethylmaleimide. Alternatively, the inhibitor can be a low-affinity antibody present in amount effective to block vesicle fusion in the composition. Similarly in the case of viral fusion proteins, low-affinity antibodies or specific fusion-blocking factors are present when the protein and vesicles are combined in the vesicle composition.

In this general embodiment in which fusion protein and vesicles are present in a single suspension prior to use, the fusion protein may be present as a soluble factor in the aqueous suspension medium of the vesicles, or may be attached to the surfaces of the vesicles, or a subpopulation thereof. In the latter case, the protein may be covalently attached directly to the vesicle surfaces, or attached covalently to a hydrophilic polymer cain, such as a polyethyleneglycol chain, which is itself coupled to a lipid vesicle lipid. Methods for direct covalent attachment of proteins to liposomes are well known, as are lipid conjugates containing a hydrophilic polymer.

In some case, particularly viral fusion proteins, the protein itself may have a hydrophobic transmembrane region which allows the protein to be anchored in he lipid vesicles.

C. Applications

The vesicle-fusion protein composition is designed for use in delivering an agent or compound to a target cell, either at an *in vivo* site or to cultures cells *in vitro*. The delivery is accomplished by fusion of the vesicles with the plasma membrane of the target cells, in the presence of the fusion protein. Several applications are discussed below.

1. Introducing Genetic Material into Cells *In Vitro*

Recombinant DNA techniques routinely involve introduction of heterologous genes into animal or plant cells in culture. In this general application, the genetic material to be transferred is encapsulated within fusogenic vesicles constructed according to the method. The vesicles are added to the recipient cells in the presence of a fusion protein, with vesicle fusion to the

cells leading to direct introduction of the encapsulated material into the cells.

2. Gene Therapy

For gene therapy uses, fusogenic vesicles containing encapsulated therapeutic genes are administered, *e.g.*, intravenously, for targeting to cells lacking the replacement gene carried in the vesicles. The vesicles may be surface modified for targeting to the cells of interest, as described above. The fusion protein may be administered separately, for migration to the target site independent of the vesicles, or may be co-administered as a single suspension with the vesicles, or may be attached to the vesicles. In the latter case, vesicle fusion is prevented during vesicle storage by the presence of a soluble inhibitor. After administration, dilution and clearance of the inhibitor in the bloodstream allows vesicle fusion with target cells to occur at the target site.

C. Addition of Membrane Factors to Target Cells

A unique therapeutic application provided by the invention is the ability to introduce cell membrane factors, *e.g.*, ion channel proteins, ligand-specific glycoproteins, and various receptor proteins into the membrane of target cells. In this application, the therapeutic vesicles are prepared with the cell membrane factor incorporated into the vesicle bilayer membranes. Vesicles of this type may be constructed either by diffusing membrane proteins into lipid vesicles, *e.g.*, in the presence of a surfactant, or by forming adding the membrane factor to either the lipid film or aqueous hydration medium used in forming the vesicles.

Administration of the vesicles and fusion protein is as described above; that is, the fusion protein is administered either separately, or together with the

vesicles in a suspension also containing a fusion-protein inhibitor. The membrane factors in the vesicle membranes are incorporated into target cell membrane with vesicle fusion to the target cells.

4. Direct Compound Delivery to Target Cells

As noted above, a variety of therapeutic compounds, including generally charged compounds, peptides, and nucleic acids, may have limited therapeutic applications because of the problem of low uptake into target cells. Using the vesicle composition of the present invention, entrapped therapeutic compounds can be delivered to target cells with high uptake via vesicle-cell fusion. Administration of the vesicles and fusion protein is as described above.

V. Mixed Vesicle Composition

The mixed-vesicle composition of the invention includes two separate populations of fusogenic vesicles, each containing a reagent or reagents capable of interacting with the reagent or reagents present in the other population. The purpose of the two-component vesicle composition is to form fused vesicles that contain both reagents, where for a variety of reasons, it is advantageous to combine the two reagents under selected conditions. The composition may contain vesicles capable of fusing under non-catalyzed conditions, *i*.e., in the absence of a vesicle fusion protein, or may contain the two populations of vesicles in combination with a vesicle-fusion protein.

A. Non-Catalyzed Components

In this embodiment, the two populations of vesicles have compositions which allow fusion in the absence of a vesicle-fusion protein. Two exemplary lipid compositions are the ones employed in the vesicle-fusion assays

described in Section II. As will be recalled, one population of the vesicles in these assays contained equimolar ratios of plasmenyl PE and PC, and the second, PS alone. More generally, the first population contains at least 10 mole percent, preferably 30-70 mole percent, of a plasmenyl glycerol lipid with a small-volume polar head group, such as plasmenyl ethanolamine. The second population contains a highly fusogenic lipid composition, such as one containing 50-100 mole percent PS or other glycerol lipid, such as diacyl glycerol, capable of forming an inverted hexagonal phase (H_{II}).

Other lipid compositions may be suitable. For example, in the assays described in Section II, vesicles formed with equimolar amounts of PE and PC, particularly PE having acyl chains of 18 carbon atoms or more, were found to be fusogenic with PS vesicles.

Each vesicle component is formed as above with an encapsulated reagent or reagent entrapped in the vesicle bilayer phase. The vesicles may be sized and otherwise processed, *e.g.*, to remove non-entrapped material, as detailed above.

Representative applications of the two-vesicle system are discussed in Section V-C below. In general, the two vesicle populations are mixed under conditions which lead to rapid vesicle fusion, *e.g.*, 1-5 seconds, although some applications will not require rapid vesicle fusion, and thus may employ lipid compositions and/or fusion conditions that lead to vesicle fusion only after a period of several minutes or longer.

B. <u>Catalyzed Fusion Components</u>

In this second embodiment, the two populations of vesicles have compositions which allow rapid fusion only in the presence of a vesicle-fusion protein. The lipid compositions described in Section III are exemplary, particularly for use with the GAPDH isoform protein. More

generally, for use with this protein, the vesicles may each be composed of between 20-40 mole percent plasmenyl glycerol lipid with a small-volume polar head group, 3-10 mole percent phosphatidylserine, 30-50 mole percent cholesterol, and 20-40 mole percent phosphatidylcholine.

The two populations of vesicles are formed as above with an encapsulated reagent or reagent entrapped in the vesicle bilayer phase. The vesicles may be sized and otherwise processed, e.g., to remove non-entrapped material, as detailed above.

In the applications discussed in Section V-C below, the catalyzed-fusion system has the advantages that (i) vesicle fusion and thus reagent mixing occurs at catalytic rates, e.g., within a msec, and (ii) both vesicle populations may have the same lipid composition.

C. Applications

1. Kinetic Studies. It is typically necessary, when conducting kinetic studies of enzymes or other biological systems, to mix the interacting components at a well defined time point. For example, ATP utilizing systems can be studied kinetically by photolytic cleavage of ATP releasing molecules, to achieve precisely timed introduction of ATP into the system. Similar kinetic studies require precise introduction of protons to an enzyme mixture, and such studies have been carried out heretofore with photolytically generated proteins.

In the present application, the biological system, e.g., enzyme, and substrate, e.g., ATP or protons, are packaged in the two vesicle populations and brought together under rapid mixing conditions, preferably employing a catalyzed fusion reaction. Reaction events are then followed by suitable reporter means, e.g., change in florescence signal.

Since vesicles in this system will fuse at different rates, it may be necessary to study changes induced by a

fusion event involving a single vesicle from each population. Ideally, the vesicles used in single-vesicle studies of these types would employ large unilamellar vesicles, in the size range typically from 2-20 microns, which can be formed by known methods, e.g., reverse evaporation phase vesicle formation. Single fusion events can be monitored in a number of ways, e.g., by fluorescence microscopy. Large vesicle fusion may require fusion protein catalysis for achieving reasonable fusion rates.

2. Microassays. A related application uses the vesicle fusion system for biochemical assays, particularly where the assay can be carried out on a micro scale. By way of example, a body-fluid sample, such as a serum-sample, contained is a fusogenic lipid vesicle is added to vesicle(s) containing assay reagents in a micro-scale assay device under vesicle-fusion conditions. After fusion, the presence or absence of analyte in the sample can be monitored by a color or fluorometric change in the fused vesicle(s), or the presence of electrochemical species, such as H_2O_2, produced in the vesicle(s) and measurable by known microelectronic devices.

3. Drug Loading. For many drugs, drug loading efficiency and stability in lipid vesicles is limited by drug diffusion from the vesicles after loading. One approach to this problem is to selectively precipitate the drug once it has been loaded. This may be done, in accordance with the present invention, by fusing drug-loaded vesicles with a second population of vesicles containing a drug-precipitating agent, such as a divalent metal, or other complexation agent, or a buffering agent effective to lower the pH of the fused vesicles.

4. Drug Activation. Some drugs can be loaded and/or stored advantageously in lipid vesicles in an inactive, prodrug form. Just prior to use, the loaded compounds can be converted to an active form by fusing

vesicles containing the prodrug with vesicles containing an activating agent, e.g., an esterase or protease enzyme. Alternatively, one or both of the different reagents may include small organic molecules which, when brought together, produce an active-drug complex, or undergo a chemical reaction leading to an active drug.

The following examples illustrate various methods for preparing fusogenic vesicles, a fusion protein, a fusion-protein inhibitor, and methods of demonstrating the fusogenic properties of the vesicle compositions. The examples are intended to illustrate the invention, but in no way limit its scope.

Materials

Bovine brain ethanolamine glycerophospholipids, 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoserine, I-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine and 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoethanolamine were purchased from Avanti Polar Lipids (Birmingham, AL). Cholesterol was obtained from Nu Chek Prep (Elysian, MN). Octadecylrhodamine (R18), Tb(Cl)3, and dipicolinic acid (DPA) were purchased from Molecular Probes (Eugene, OR). DE-52 anion exchange resin was purchased from Whatman (Maidstone, EN). DTT was purchased from Calbiochem (San Diego, CA), and GTP and GMP were purchased from Beckman (Indianapolis, IN). Multiphor 2-D electrophoresis supplies including Immobilines II were purchased from Pharmacia Biotech Inc. (Piscataway, N.J.). HPLC-grade solvents were purchased from Baxter Scientific (McGaw Park, IL). Most other chemicals were obtained from Sigma (St. Louis, MO).

Phospholipids were purified to remove trace contaminants on an Altex Ultrasphere-Si column (4.6 mm × 25 cm) utilizing a mobile phase comprised of hexane/isopropanol with a 1-7% H_2O gradient (Geurts van Kessel, *et al.*, 1977).

Plasmenyl ethanolamines (16:0-18:1) plasmenyl ethanolamine and 18:0-20:4 plasmenyl ethanolamine) were synthesized as described previously (Glaser and Gross, 1994; Han, *et al.*, 1992), and as detailed in Example 1A. Octadecylrhodamine was purified on an Altex Ultrasphere CN column (4.6 nm × 25 cm) utilizing a mobile phase comprised of acetonitrile and a gradient of triethanolamine (20 mm to 50 mm, pH 7.0 with acetic acid) over 40 ml at a flow rate of 2.0 ml/min. Purified R18 fractions were extracted using a modified Bligh and Dyer and were stored under nitrogen at -20°C in chloroform.

Example 1

Preparation of Plasmenyl Ethanolamine

A. Synthesis of Plasmenyl Ethanolamine

Bovine brain ethanolamine glycerophospholipids (1 g/100 mL chloroform) were subjected to alkaline methanolysis by mixing with 0.5 N methanolic NaOH (100 mL) for forty-five minutes at 25°C. The reaction was stopped by addition of 50 mL of 1 N acetic acid and placed in a separatory funnel. After gentle mixing, the lower phase was collected and dried under vacuum at room temperature. The resultant yellow paste was dissolved in 6 mL of chloroform, filtered, and injected onto a Dynamax Preparatory Si HPLC column (31.4 mm × 20 cm) previously equilibrated with chloroform. After 20 minutes of elution with chloroform at a flow rate of 10 mL\min., a linear gradient to 100% methanol over 100 min. was initiated. Lysoplasmenyl ethanolamine eluted at 60% methanol, was detected by TLC (Whatman LKG plates) using another mobile phase (system A, 95:35:6 chloroform\methanol\water, v\v\v) by subsequent iodine staining (lysoplasmenyl ethanolamine R_f=0.17).

Lysoplasmenyl ethanolamine-containing fractions were pooled, dried under nitrogen, and resuspended in chloroform at a yield of 80% of theoretical. The ethanol

amine head group was then blocked using a N-9-fluorenylmethoxylcarbonyl (Fmoc) protection group. Protection of the primary amine was affected by stirring 170 mg of lysoplasmenyl ethanolamine (0.37 mmol) in distilled $CHCl_3$ (distilled over P_2O_5) with a 5-fold molar excess of succinimidylfluroenylmethoxyl-carbodiimide (600 mg, 1.85 mmol) and 240 mg (3.7 mmol) of imidazole for four hours at room temperature. A second addition of 240 mg of succinimidyl-Fmoc (0.74 mmol) and 100 mg of imidazole (1.48 mmol) was performed, and the reaction was stirred overnight at 25°C prior to the separation of the reaction products by the method of Bligh and Dyer (1959).

Purification of Fmoc-lysoplasmenyl ethanolamine was accomplished using straight phase HPLC employing a chloroform\methanol linear gradient as described above, with Fmoc-LPE eluting at 20% methanol. Fractions containing Fmoc-LPE (R_f=0.29 in TLC neutral system A) were pooled, dried under vacuum, resuspended in chloroform for storage, and stored under an atmosphere of nitrogen.

50 mg (0.072 mmol) of Fmoc-lysoplasmenyl ethanolamine was dried under nitrogen and resuspended in chloroform (2 mmol) under anhydrous conditions, and, after addition of recrystallized DMAP (10 mg, 0.082 mmol), the appropriate fatty acid anhydride or acyl chloride was added in 4-10-fold molar excess (100-200 mg) and stirred for 6 hours at 35°C.

Dicyclohexylcarbodiimide (in 1-mg iterative additions) was added to reactions to regenerate the anhydride every two hours. The reaction was quenched by addition of one-quarter volume of methanol (1 mL) and neutral deprotection of the Fmoc group was accomplished by addition of one-quarter volume of diethylamine and subsequent stirring for eight hours at room temperature.

The resultant plasmenyl ethanolamine was purified after Bligh and Dyer extraction with a Dynamax Preparatory Si HPLC column (21.4 mm × 25 cm) previously equilibrated

with hexane\isopropanol\water\ammonium hydroxide (48:48:4:0.005,v\v) as the mobile phase employing a 4-8.5% H_2O gradient, as described in Blank & Snyder (1983). Fractions containing plasmenyl ethanolamine were pooled and further purified on an Altex Ultrasphere-Si column (4.6 mm × 25 cm) with a mobile phase of hexane\isopropanol (50:50, v\v) with a 1-7% H_2O gradient (Geurts van Kessel, *et al.*, 1977). A total of 27 mg of arachidonoylated plasmenyl ethanolamine was obtained. Individual molecular species of plasmenyl ethanolamine (either 16:0, 18:0, or 18:1 at the *sn*-1 position) were resolved on a Beckman C18 reverse-phase HPLC column (4.6 mm × 25 cm) using a mobile phase comprised of methanol\acetonitrile\water (90.5:2.5:7,v\v) containing 20 nM choline chloride as described previously by Gross (1984). The purity of plasmenyl ethanolamine was assessed by TLC employing neutral system A, a base system (65:25:5 chloroform\methanol\ammonium hydroxide), and an acid system (6:8:2:2:1 chloroform\acetone\methanol\acetic acid\water). The concentration of phospholipids was quantified by capillary gas chromatography following derivatization by acid methanolysis as described previously by comparisons with internal standard.

B. <u>Preparation of Plasmalogen-Depleted Bovine Brain Ethanolamine Glycerophospholipids</u>

Plasmenyl ethanolamine was selectively removed from total bovine brain ethanolamine glycerophospholipids by exploiting the acid lability of the vinyl ether linkage.

Bovine brain ethanolamine glycerophospholipids (15 mg) were dried under nitrogen and subsequently exposed to HCl fumes for 25 minutes. After two minutes of flushing with nitrogen, the sample was resuspended in chloroform, and plasmalogen-depleted PE was isolated on an Altex Ultrasphere-Si column (4.6 mm × 25 cm) using a mobile phase of hexane\isopropanol (50:50 v\v) with a 1-7% H_2O gradient. The fatty acid compositions of bovine brain PE

in plasmalogen-depleted bovine brain PE were quantified by capillary gas chromatography after acid methanolysis. The results are found in Table III below.

Table III

Fatty Acid and Aldehyde Composition of Ethanolamine Glycerophospholipids

Depleted Carbon Chain Glycerophospholipids	Bovine Brain Ethanolamine Glycerophospholipids	Plasmalogen -Bovine Brain
plasmal 16:0	6.5 ± 0.8	0.5 ± 0.3
acyl 16:0	3.5 ± 0.3	6.8 ± 0.7
plasmal 18:0	11.1 ± 0.5	1.3 ± 0.2
acyl 18:0	13.0 ± 0.4	33.1 ± 2.0
plasmal 18:1	15.6 ± 1.0	0.5 ± 0.7
acyl 18:1	17.6 ± 0.9	22.6 ± 0.6
acyl 20:4	13.4 ± 0.7	12.5 ± 0.6
acyl 22:4	6.6 ± 1.3	5.7 ± 0.1
acyl 22:5	4.1 ± 0.7	5.1 ± 1.3
acyl 22:6	8.4 ± 0.4	10.3 ± 2.0

Example 2

Vesicle-Fusion Assays

A. Octadecyl (R_{11}) Fusion Assay

Phospholipids used for fusion assays were co-dissolved with lipid fluorescent probes in chloroform, evaporated under nitrogen, and evacuated for one hour at 100 mTorr. Multilamellar lipid vesicles were formed by resuspension of liposome buffer A (100 mM $NaCl_2$, 5 mM Na HEPES, 0.1 mM EGTA, pH 7.4) and vigorous vortexing. Small unilamellar vesicles (SUVs) were formed by sonicating the multi-lamellar lipid vesicles for five minutes at 46°C

using a 40% duty cycle at a power level of 1.5 with a Vibra Cell sonicator equipped with a medium tip.

All lipid vesicles were used immediately after preparation and were maintained under a nitrogen atmosphere during all steps in the procedure. The octadecyl (R_{18}) fusion assay was performed as in Hoekstra, *et al.*, in 1984, with the following modifications. Phosphatidylserine vesicles were prepared with 4% R_{18} and were mixed with an equal molar amount of labeled vesicles comprised of 50% 16:0-18:1 phosphatidylcholine and 50% ethanolamine glycerophospholipid. This mixture was loaded into one chamber of an SLM-Amino spectroflurometer equipped with an SLM stopped-flow apparatus (model no. FP-052). The other chamber was loaded with liposome buffer alone or with liposome buffer containing an addition, 20 mM $CaCl_2$. The contents of the chambers were rapidly mixed (dead time 7 ms) in a 1:1(v/v) ratio.

Fusion was monitored by the temporal dependence of R_{18} dequenching observed at 590 nm after excitation at 560 nm. The final total lipid concentration in assays was approximately 200 micromolar. The 0% fusion level was assessed by monitoring fluorescence when the vesicles were mixed at 0 mM $CaCl_2$. The 100% fusion level was measured by preparing lipid vesicles comprised of the mixture which would result if all vesicles fused and subsequently quantifying the resultant R_{18} fluorescence. Fluorescence readings were normalized using the 0% and 100% fusion levels and were expressed as a percentage of maximum fusion (F_{max}). The reported initial rates (expressed in terms of $F_{max\%}$ $^{s-1}$) represent predominantly fusion rates under the conditions employed since the high Ca^{2+} concentration used in conjunction with the use of SUVs each predispose to membrane fusion, Wilschut, *et al.* (1980).

B. NBD-PE/Rh-PE Assay

This assay was performed by preparing phosphatidylserine SUVs containing 1.8% Rh-PE and 1.2% NBD-PE and adding an equimolar amount of unlabelled PC/PE vesicles pursuant to (Struck, *et al.* (1981); and Hoekstra, 1982). Fusion was monitored by NBD-PE fluorescence at 530 nm after excitation at 464 nm. The 100% fusion level was assessed by preparing lipid vesicles composed of 46.5% PS, 25% PC, 25% PE, 0.9% Rh-PE, and 0.6% NBD-PE and subsequently quantifying fluorescence intensity.

Since Rh-PE partially quenches NBD-PE fluorescence by resonance energy transfer at the initial concentrations used in these experiments, membrane fusion results in an increase in NBD-PE fluorescence as available membrane surface area increases.

C. Contents Mixing Assay

Vesicles for use in a contents mixing assay were prepared by first resuspending phospholipids in either 20 mM $NaCl_3$ 50 mM DPA, and 5 mM Na HEPES (pH 7.4) pursuant to Wilschut and Papahadjopoulos (1979); Wilschut, *et al.* (1980), Düzgüneş, *et al.* (1987).

SUVs containing entrapped $TbCl_3$ or DPA were separated from unencapsulated probe by gel filtration chromatography employing a Sepharose 6B column equilibrated with 100 mM NaCl, 5 mM Na HEPES, and 1.0 mM EDTA, pH 7.4. An aliquot of the vesicle-containing fraction was subjected to Bligh and Dyer extraction, acid methanolysis, and capillary gas chromatography to quantify the liposome lipid concentration for subsequent fluorescence assays. Contents-mixing assays were performed by incorporating the DPA probe into PS SUVs and Tb probe into PC/PE SUVs. After mixing in equal molar concentrations and loading into the stopped-flow apparatus, fusion was monitored through the formation of fluorescence Tb/DPA complex measured at > 470 nm after excitation at 216 nm. Final

total lipid concentration in the assay were approximately 200 μM. The 100% fusion level was assessed by measuring the fluorescence of vesicles prepared in a buffer of 10 mM NaCl, 25 mM sodium citrate, 1.25 mM $TbCl_3$, 25 mM DPA, and 5 mM Na HEPES (pH 7.4) and processed as described above. All fusion assays were performed at 37°C.

Example 3

Plasmenyl Ethanolamine-Mediated Vesicle Fusion

A. Purified Plasmenyl Lipids

Small unilamellar vesicles (SUVs) composed of equal molar ratio of choline and various purified ethanolamine glycerophospholipids, including plasmenyl ethanolamines, were added in equal parts to PS SUVs containing R_{18}, prepared as described in Example 2. The rate of liposome fusion was quantified by stop-flow kinetics through measurement of the increased R_{18} fluorescence intensity, which reflects its attenuated quenching as the effective membrane and surface area of the probe distribution increases after fusion with an unlabeled vesicle, with the results shown in Fig. 1, and discussed above.

To verify the differences in observed fusion rates were not due to selective breakdown of one subclass of ethanolamine glycerophospholipid, aliquots of lipid vesicles employed in the fusion studies were extracted by the method of Bligh and Dyer and analyzed by TLC and straight-phase HPLC. No lysophospholipids or other contaminants were detected within the time frame of the assay. For higher sensitivity detection, 16:0-18:1 plasmenyl ethanolamine was synthesized with a ^3H label on the *sn*-2 oleoyl group. This label was added to a liposome preparation containing unlabeled PC and PE. No significant generation of radiolabeled fatty acid or lysophospholipid was observed during the procedures employed.

B. Plasmalogen-Depleted Bovine Brain PE

Plasmalogen-depleted bovine brain PE were prepared as described in Example 1B. No significant changes were observed in the percentages of polyunsaturated fatty acids at the *sn*-2 position in this function.

Small unilamellar vesicles (SUVs) composed of equal molar ratio of phosphatidylcholine and bovine brain PE or POPC/plasmalogen-depleted bovine brain PE were added in equal parts to PS SUVs containing R_{18}, as above. The rate of liposome fusion was quantified by stop-flow kinetics through measurement of the increased R_{18} fluorescence intensity, with the results shown in Fig. 2, and discussed above.

C. NBD-PE/Rh-PE Assay

To verify the results obtained with the R_{18} fusion assay, a second lipid-mixing assay utilizing NBD-PE and Rh-PE was used. Phosphatidylserine (PS) SUVs containing 1.8% Rh-PE and 1.2% NBD-PE were mixed with equimolar amounts of unlabelled PC/PE vesicles, where the PE species was either plasmenyl ethanolamine (16:0-18:1), brain PE, or phosphatidylethanolamine (PE). The fusion kinetics were examined as described in Example 2B, with the results shown in Fig. 3, discussed above.

D. Contents Mixing Assay

Since the R_{18} fusion assay does not discriminate entirely between membrane apposition and *bona fide* membrane fusion, the conclusion that plasmenyl ethanolamine molecular species facilitate membrane fusion is further substantiated by quantitation of internal contents mixing.

To assess membrane fusion rates, the interior of PCFS and PC/PE SUVs (equimolar mixtures of PC/PE for both 16:0-18:1 plasmenyl ethanolamine and 16:0-18:1) were loaded with dipicolinic acid and Tb^{3+}, respectively, and the rates

of mixing of internal contents were quantified on the basis of the increase in fluorescence intensity resulting from the formation of Tb/DPA complex, with the results shown in Fig. 4.

Example 4

Preparation of GAPDH Isoform

A. Measurement of Glyceraldehyde 3-Phosphate Dehydrogenase Activity

The activity of GAPDH was measured spectrophotometrically utilizing modifications of the methods of Cori, *et al.* (1948) and Steck, *et al.* (1973). Briefly, sample was added to a 1 cm path-length semi-micro cuvette containing 50 mM triethanolamine (pH 7-6), sodium arsenate (50 mM) (pH 8.8), 2.4 mM glutathione (reduced), 0.5 mM NAD, and water in a final volume of 990 μl. After establishing a baseline (1 min), the reaction was initiated with 10 μl of 10 mM D-glyceraldehyde 3-phosphate (prepared according to Sigma product bulletin G-8007) and absorbance at 340 nm was measured for 5 minutes. Units of GAPDH activity represent the mass (μmol) of D-glyceraldehyde 3-phosphate converted per minute. The mass of NADH generated was calculated utilizing ϵ = 0.622.

B. Purification of the Membrane Fusion Protein from Rabbit Brain Cytosol

New Zealand White rabbits (typically, ten per preparation) were sacrificed by cervical dislocation and brains were harvested and placed in ice-cold homogenization buffer (30% w/v) consisting of 250 mM sucrose, 30 mM Tris·Cl, 10 mM EGTA, 2 mM EDTA, and 1 mM DTT, (pH 7.4, measured at 25°C). Brains were homogenized using three 10 s pulses from a Brinkman PT 10/35 Polytron apparatus at incremental output settings of 4.5, 5, and 5.5. The homogenate was initially centrifuged at 10,000 × g for 20 minutes and the resultant supernatant was centrifuged at 100,000 × g for 60 minutes. The crude

cytosol was twice dialyzed for 6 hours against 500 volumes of buffer A (50 mM Tris·Cl), 0.1 mM EGTA, 0.1 mM EDTA, and 1 mM DTT, pH 7.0 at 4°C). The dialyzed cytosol was loaded onto a DE-52 column (2.6 cm × 20 cm) previously equilibrated with buffer A at a flow rate of 2 ml/minute. Fractions from the DE-52 column were assayed for their ability to catalyze membrane fusion.

Activity was quantified in U/ml where U represents the nmol of lipid fused per second in an assay containing 200 μM lipid in a final volume of 100 λ. Fractions from the void volume containing fusion-catalyzing activity were pooled, filtered utilizing a Millipore GS 0.22 μm filter, and loaded onto a HiLoad SP Sepharose column (1.6 cm × 10 cm, Pharmacia) previously equilibrated with 20 mM Tris·Cl, 0.1 mM EGTA, 0.1 mM EDTA, and 1 mM DTT, (pH 7.0 at 4°C) (buffer B) at 3.0 ml/minute. After washing with 3 column volumes of buffer B, membrane fusion activity was eluted utilizing a nonlinear sodium chloride gradient from 0 to 0.5 M NaCl in buffer B. Active fractions from the HiLoad SP Sepharose column were pooled, diluted 3-fold with buffer B, and loaded onto a GTP-agarose column (Sigma, G-9768, Lot 70H9545, 1 cm × 5 cm) at a flow rate of 0.3 ml/minute. After washing the affinity matrix with 5 column volumes of buffer B, bound proteins were sequentially eluted with 5 mM GMP in buffer B, 3 mM GTP in buffer B and, finally, an additional 5 column volumes of buffer B alone. Membrane fusion activity was eluted with 20 mM tetrasodium tripolyphosphate and 10 mM NAD^+ in buffer B. Column eluents were assayed for GAPDH activity and membrane fusion activity.

GTP-agarose affinity eluents were further purified either by reverse phase HPLC (in preparation for protein sequencing) or by Mono Q chromatography. Anion exchange chromatography was accomplished utilizing a Mono Q column equipped for a Smart System FPLC (Pharmacia). Active fractions were pooled, diluted 10-fold into buffer C (20

mM Tris·Cl, 0.1 mM EGTA, 0.1 mM EDTA, and 1 mM DTT, pH 8.5 at 4°C) and loaded onto a Mono Q PC 1.6/5 column previously equilibrate buffer C at a flow rate of 200 μl/minute. Fusion-catalyzing activity was eluted using a continuous gradient from 0 to 500 mM NaCl over 6 ml. Aliquots of column eluents were assayed for membrane fusion activity or subjected to [^{125}I]-Boulton-Hunter labeling previously described (Hazen, *et al.*, 1990).

The GTP affinity column active fraction (0.1 ml) utilized for sequencing was diluted 1:1 with buffer containing 14% acetonitrile and 0.2% trifluoroacetic acid and loaded onto a C18 HPLC column (Vydac, 300 Å pore size, 4.6 mm × 25 cm) pre-equilibrated with 10% mobile phase B at 500 μl/minute (mobile phase A: water containing 0.1% TFA; mobile phase B: 70% acetonitrile and 0.12% TFA). Proteins were eluted with a gradient of 10 to 70% mobile phase B over 50 minutes at a flow rate of 500 μl/minute. Homogeneity of the 38 kDa band eluting as the major uv absorbing peak from the RP-HPLC was verified by SDS-PAGE prior to submission for protein sequencing.

C. Two-Dimensional Electrophoresis

Two-dimensional electrophoresis was performed utilizing a Multiphor system (Pharmacia) with the first dimension isoelectric phast-gels cast utilizing Immobilines II in a 4% polyacrylamide matrix containing a pH gradient from pH 7.0 to 10.0 over 10 cm. Following polymerization, washing and drying of the gel, 3 mm strips were cut and reswelled in buffer containing 8 M urea, 1% NP-40 (Pierce SurfactAmps), 1 mM DTT, and 0.25% Pharmalyte 7-9 for 24 hours. Protein samples were diluted into buffer containing 10 M urea, 1% NP-40, 0.1% β-mercaptoethanol, and 0.25% Pharmalyte 7-9 and were concentrated utilizing Microcon-10 ultrafiltration units. First dimension gels were electrophoresed for 26 hours using a discontinuous voltage gradient (5 hours at 500 V,

18 hours at 2500 V and 2.5 hours at 3500 V). After equilibration of 1-D gels with SDS-PAGE buffer, samples were electrophoresed on either ExcelGel SDS 8-18% or ExcelGel SDS Homogeneous 12.5% gels (Pharmacia Biotech Inc.). Gels were subsequently either silver stained (BioRad Silver Stain Plus system) or transferred to PVDF paper for subsequent Western blotting.

Example 5

Preparation of Anti-G6PDH of Monoclonal Antibodies

Four mice were initially injected with purified membrane fusion protein from the GTP-agarose column, and subsequent booster injections were made with rabbit muscle GAPDH. Hybridomas were formed from mouse spleen as described previously (Harlow & Lane, 1988) and supernatants were screened for anti-GAPDH antibodies by automated particle-concentration fluorescence immunoassays. Samples possessing activity after the initial screen were verified by Western blot analysis of rabbit brain cytosol. Hybridomas were used to generate ascites fluid by traditional methods (Harlow & Lane, 1988) and the resultant. IgG was purified from ascites fluid utilizing Protein-A agarose affinity chromatography.

Example 6

Preparation of Tubulin Protein

A. Preparation of Tubulin

New Zealand rabbit brains were harvested after cervical dislocation and placed [30% (w/v)] in ice-cold homogenization buffer (250 mM sucrose, 30 mM Tris-Cl, 10 mM EGTA, 2 mM EDTA and 1 mM DTT, (pH 7.4, measured at 25°C). Homogenization was performed utilizing three 10 sec pulses from a Brinkmann PT 10/35 polytron at incremental power settings of 4.5, 5, and 5.5. The homogenate was centrifuged at 10,000 × g for 20 min and the supernatant was reserved. Subsequent centrifugation of the

supernatant at 100,000 × g for 60 min yielded crude cytosol, which was twice dialyzed against 500 volumes of buffer A (50 mM Tris-Cl, 0.1 mM EGTA, 0. 1 mM EDTA, and 1 mM DTT, pH 7.0 at 4°C).

Dialyzed cytosol was loaded onto a DE-52 column (2.6 cm × 20 cm) previously equilibrated with buffer A at a flow rate of 2 ml/min. Adsorbed proteins were eluted utilizing a 0-400 mM NaCl gradient in buffer A over 400 ml. The inhibitory potency of column eluents was assessed after appropriate dilution (as indicated in the figure legends) by quantifying the differences in percent of GAPDH isoform-catalyzed membrane fusion in the presence relative to the absence of diluted aliquots of column eluents. Fractions containing the highest levels of inhibitory activity were pooled, dialyzed against 100 volumes of buffer B (20 mM Tris-Cl, 0.1 mM EGTA, 0.1 mM EDTA and 1 mM DTT, (pH 7.0 at 4°C)) and loaded onto a Mono Q column previously equilibrated with buffer B. Adsorbed proteins were eluted utilizing a nonlinear NaCl gradient (O Lo 600 mM NaCl in buffer B) as shown in the figure legends. To further exploit the resolving power of Mono Q chromatography, the most potent inhibitory fractions were pooled, dialyzed against buffer B, and rechromatographed on the Mono Q column utilizing a shallow gradient of NaCl in buffer B.

B. <u>Purification of Tubulin by Assembly/Disassembly Cycling</u>

Tubulin was purified by assembly/disassembly of microtubules using the method of Shelanski, *et al.*, (1979). Briefly, rabbit brain cytosol was prepared as described above and diluted (1:1, v/v) with buffer comprised of 8 M glycerol, 2 mM GTP, 200 mm NaMES, 1 mM $MgCl_2$, and 2 mM EGTA (pH 6.4). After incubation at 37°C for 20 minutes (during which time microtubule assembly occurred), microtubules were pelleted by centrifugation at 100,000 × g for 60 min at 25°C. The pellet was

resuspended in ice-cold (4°C) buffer (100 mM NaMES, 0.5 mM $MgCl_2$, and 1 mM EGTA (pH 6.4)) and dispersed using a Potter Elvehjem homogenizer and incubated at 4°C for 20 min. After microtubule disassembly, the sample was centrifuged at 100,000 × g and the supernatant collected. To the supernatant, an equal volume of buffer (8 M glycerol, 2 mM GTP, 200 mM NaMES, 1 mM $MgCl_2$, and 2 mM EGTA (pH 6.4)) was added. Microtubule formation was again accomplished by incubation at 37°C for 20 minutes and the above sequence of centrifugation and disassembly was repeated.

After the second disassembly, tubulin was further purified from microtubule-associated proteins by Mono Q chromatography. The supernatant was filtered, dialyzed against buffer C (100 mM NaMES, 1 mM EGTA, and 0.5 mM $MgCl_2$ (pH 6.6)) and loaded onto a Mono Q column previously equilibrated with buffer C. Bound tubulin was eluted utilizing a nonlinear NaCl gradient in buffer C as indicated in the figures. Tubulin-containing column eluents were twice dialyzed against a 100-fold excess of buffer D (10 mM potassium phosphate (pH 7.0) containing 0.3 mM $CaCl_2$) prior to loading onto an BioGel HPHT hydroxylapatite column (Bio Rad) (1 × 5 cm) previously equilibrated with buffer D. The column was developed utilizing a gradient of 10 mM potassium phosphate, 0.3 MM $CaCl_2$ to 350 mM potassium phosphate, 0.01 mM $CaCl_2$ in buffer D over a 30 ml volume.

C. <u>Preparation of Tubulin-Sepharose Affinity Resin and Affinity Chromatography</u>

Homogeneous tubulin (purified by the assembly/disassembly method followed by sequential Mono Q and HA chromatographies) was twice dialyzed against 100 volumes of buffer E (0.1 M $NaHCO_3$ with 0.5 M NaCl (pH 8.0)). The tubulin solution (0.1 mg/ml) was dialyzed against 100 volumes of buffer E for 15 h and was added, in equal volumes to a solution of activated CNBr-Sepharose (1

g/5 ml). After incubation for 24 h at 4°C, unreacted groups were blocked with 0.2 M glycine (pH = 8.0) for 2 hours and the tubulin-sepharose matrix was washed with repetitive alternating washes of buffer E and buffer F (0.1 M acetate, 0.5 M NaCl, pH 4.5). After equilibration of the tubulin-Sepharose column (0.9 × 5 cm) with buffer B (20 mM Tris-Cl, 0.1 mM EGTA, 0.1 mM EDTA, and 1 mM DTT, (pH 7.0 at 4°C) protein was loaded onto the column, washed with 10 column volumes of buffer B and bound proteins were eluted with a step gradient 0.5 M NaCl in buffer B.

Although the invention has been described with respect to particular formulations, compositions, and methods, it will be appreciated that various changes and modifications can be made without departing from the invention.

IT IS CLAIMED:

1. A vesicle composition for use in delivering a therapeutic agent to target biological cells comprising

artificial lipid vesicles (i) composed of vesicles-forming lipids that include at least 10 mole percent of a plasmalogen glyceryl lipid with a small-volume polar head group, and (ii) that contain the therapeutic agent in entrapped form, and

an isolated fusion protein effective to facilitate fusion of the lipid vesicles with the cells, when the cells, vesicles and protein are brought together.

2. The composition of claim 1, wherein the fusion protein and vesicles are stored as separate components prior to use.

3. The composition of claim 1, wherein the fusion protein is present in a suspension of the vesicles, and the composition further includes an inhibitor of the protein's vesicle-fusion activity.

4. The composition of claim 3, wherein the fusion protein is a glyceraldehyde-3-phosphate dehydrogenase isoform, and the inhibitor is tubulin.

5. The composition of claim 1, wherein the protein is attached to the surface of the lipid vesicles.

6. The composition of claim 1, for use in intravenous administration, wherein the vesicles have sizes in the 30-80 nm range.

7. The composition of claim 1, wherein the vesicles are composed of between 10-50 mole percent plasmenyl phospholipid selected from the group consisting of serine,

ethanolamine or hydroxyl head groups, 3-15 percent mole negatively charged phospholipid, 20-50 mole percent cholesterol, and 0-40 mole percent neutral phospholipid.

8. The composition of claim 7, wherein the vesicles are composed of between 20-40 mole percent plasmenyl ethanolamine phospholipid, 3-10 mole percent phosphatidylserine, 30-50 mole percent cholesterol, and 20-40 mole percent phosphatidylcholine.

9. The composition of claim 1, wherein said fusion protein is an isoform of glyceraldehyde-3-phosphate dehydrogenase.

10. The composition of claim 1, wherein said fusion protein is an NEM-sensitive fusion protein.

11. The composition of claim 1, wherein said fusion protein is a viral fusion protein.

12. The composition of claim 1, which further includes cell-specific targeting molecules carried on the vesicle surfaces for binding the vesicles specifically to target cells.

13. The composition of claim 1, wherein the therapeutic agent is a nucleic acid or nucleic acid analog, and the agent is encapsulated within the lipid vesicles.

14. The composition of claim 1, wherein the therapeutic agent is selected from the group consisting of a protein, glycoprotein, antibody, and receptor ligand effective to enhance a selected activity of target cells, when incorporated into target-cell membranes, wherein the therapeutic agent is present in the vesicle membranes.

15. A vesicle composition for use in delivering a therapeutic agent to target biological cells comprising

artificial lipid vesicles (i) composed of vesicles-forming lipids that include at least 10 mole percent of a plasmalogen glyceryl lipid with a small-volume polar head group, and (ii) that contain the therapeutic agent in entrapped form.

16. The composition of claim 15, wherein the vesicle-forming lipids include 30-70 mole percent plasmenyl ethanolamine phospholipid and 30-70 mole percent phosphatidylcholine.

17. The vesicles composition of claim 15, wherein the vesicles are composed of between 20-40 mole percent plasmenyl glycerol lipid with a small-volume polar head group, 3-10 mole percent phosphatidylserine, 30-50 mole percent cholesterol, and 20-40 mole percent phosphatidylcholine.

18. The composition of claim 17, which further include an isolated fusion protein effective to facilitate fusion of the lipid vesicles with target cells, when the cells, vesicles and protein are brought together.

19. Components for forming a lipid vesicle composition containing first and second reagents capable of interacting with one another comprising

first and second populations of artificial lipid vesicles containing said first and second reagents, respectively, where the first population of vesicles is composed of vesicle-forming lipids containing at least 10 mole percent of a plasmalogen phospholipid selected from the group consisting of phosphatidyl serine, phosphatidyl ethanolamine, and phosphatidic acid, and the second population of vesicles is composed of vesicle-forming

lipids effective to allow fusion with the first-population vesicles, when the two populations are brought into contact with one another.

20. The components of claim 19, wherein the vesicle-forming lipids in the first population of vesicles include 30-70 mole percent plasmenyl phospholipid and 30-70 mole percent phosphatidylcholine.

21. The components of claim 19, wherein the vesicle-forming lipids in the first population of vesicles include between 20-40 mole percent plasmenyl ethanolamine, 3-10 mole percent phosphatidylserine, 30-50 mole percent cholesterol, and 20-40 mole percent phosphatidylcholine.

22. The components of claim 19, which further include a fusion protein effective to promote fusion between vesicles in the two different populations.

23. The components of claim 22, wherein said fusion protein is an isoform of glyceraldehyde-3-phosphate dehydrogenase.

24. The components of claim 18, wherein said fusion protein is an NEM-sensitive fusion protein or a viral fusion protein.

25. A purified membrane fusion protein characterized by:

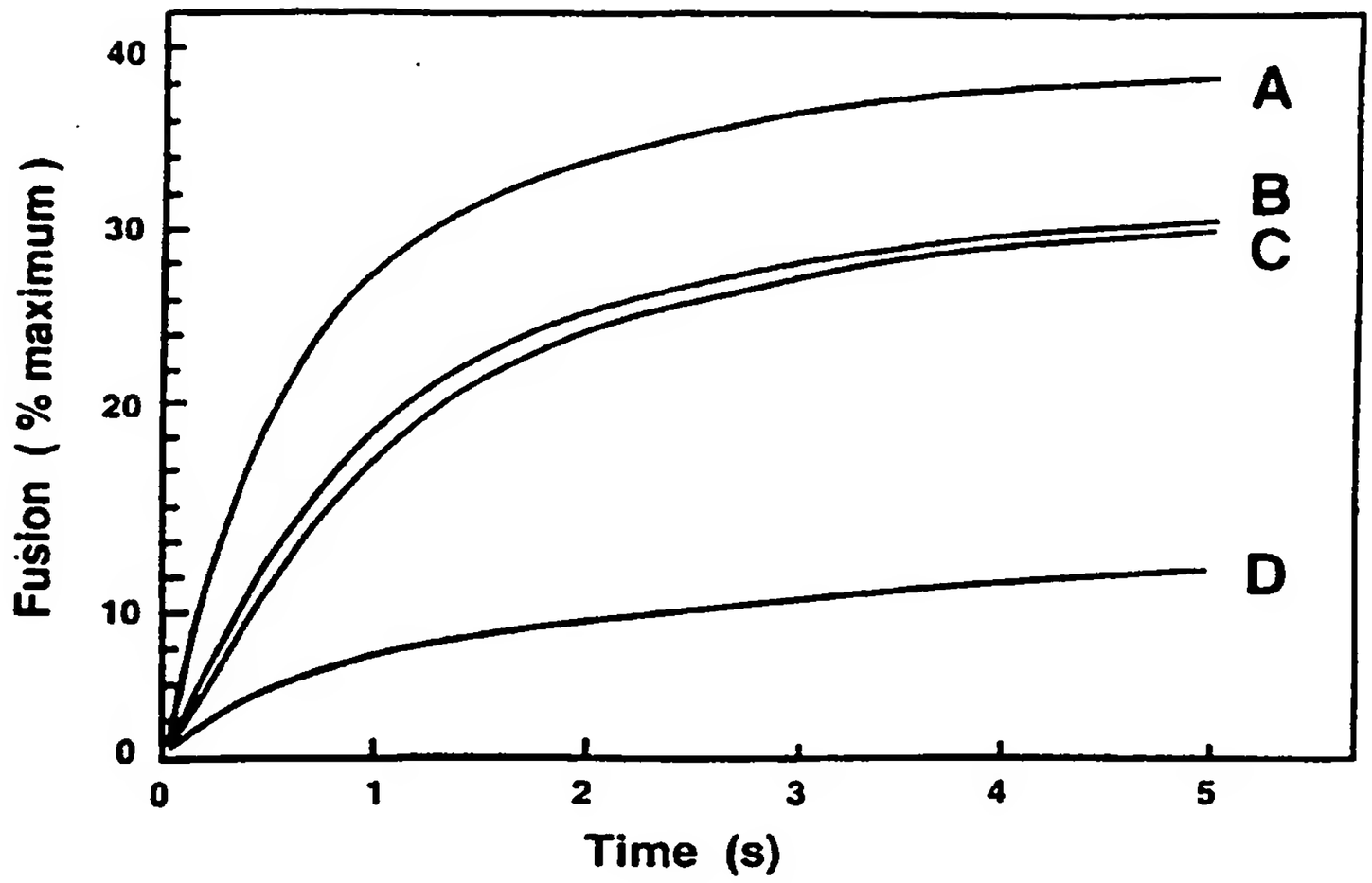
(i) a molecular weight of about 38 KDal, as determined by SDS PAGE;

(ii) binds to anti-GAPDH antibodies; and

(iii) the ability to promote rapid fusion of lipid vesicles composed of 20-40 mole percent plasmenyl ethanolamine, 3-10 mole percent phosphatidylserine, 30-50

mole percent cholesterol, and 20-40 mole percent phosphatidylcholine.

26. The use of the membrane fusion protein of claim 25 to promote fusion in lipid vesicles.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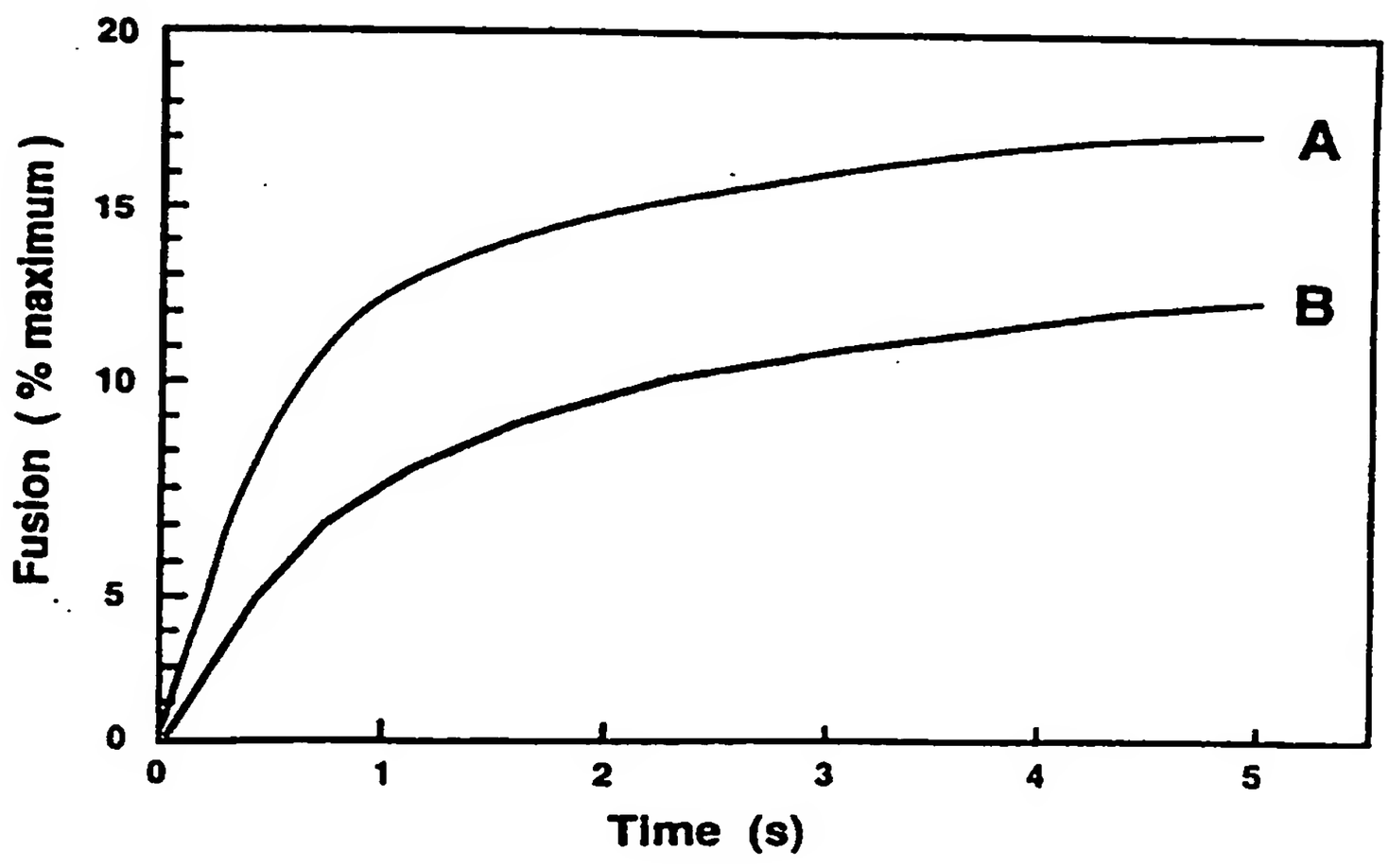
20
10
0
0
1
2
3
4
5
Time (s)
A
B
C
D

Fig. 1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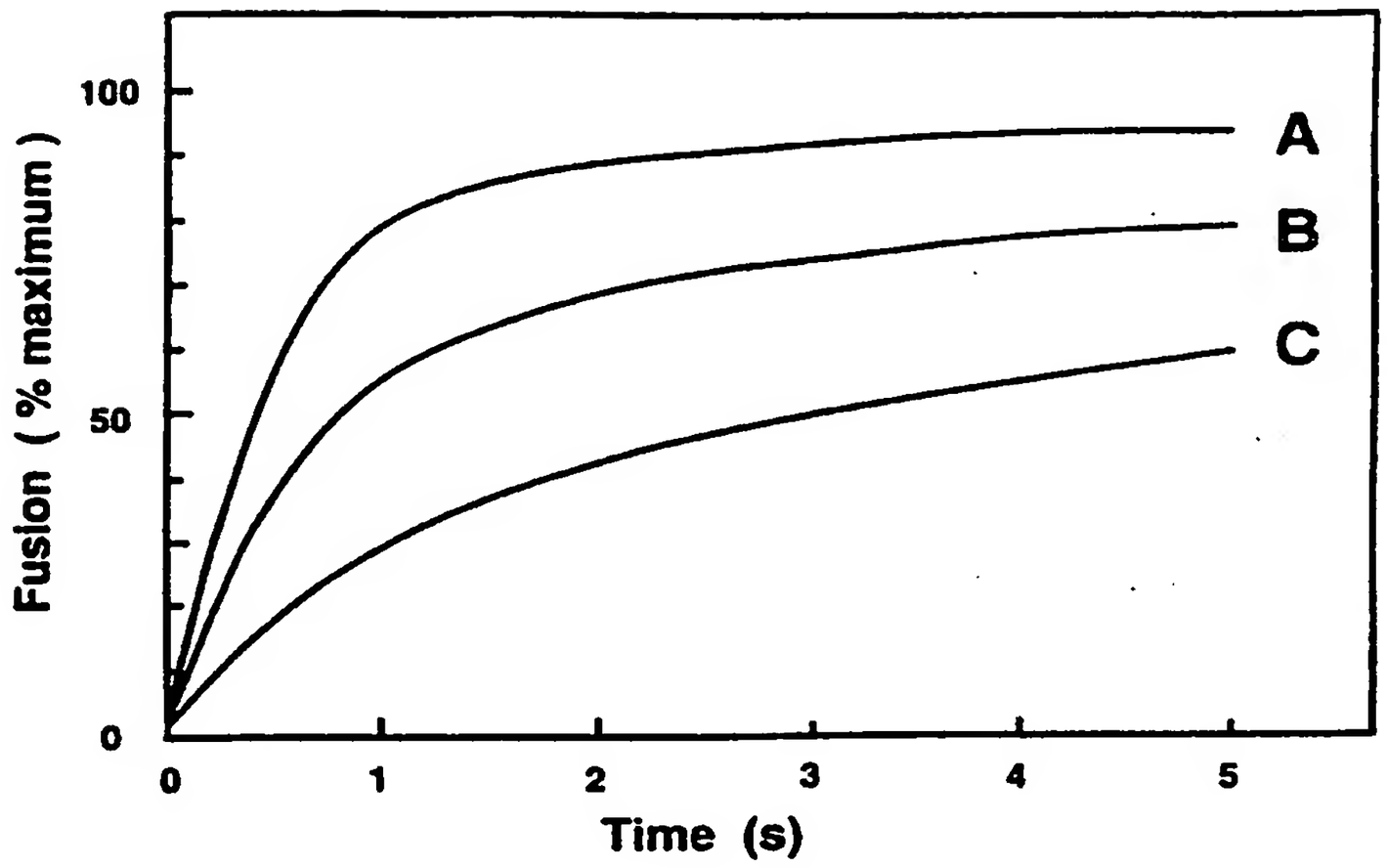
0
0
1
2
3
4
5
Time (s)
A
B

Fig. 2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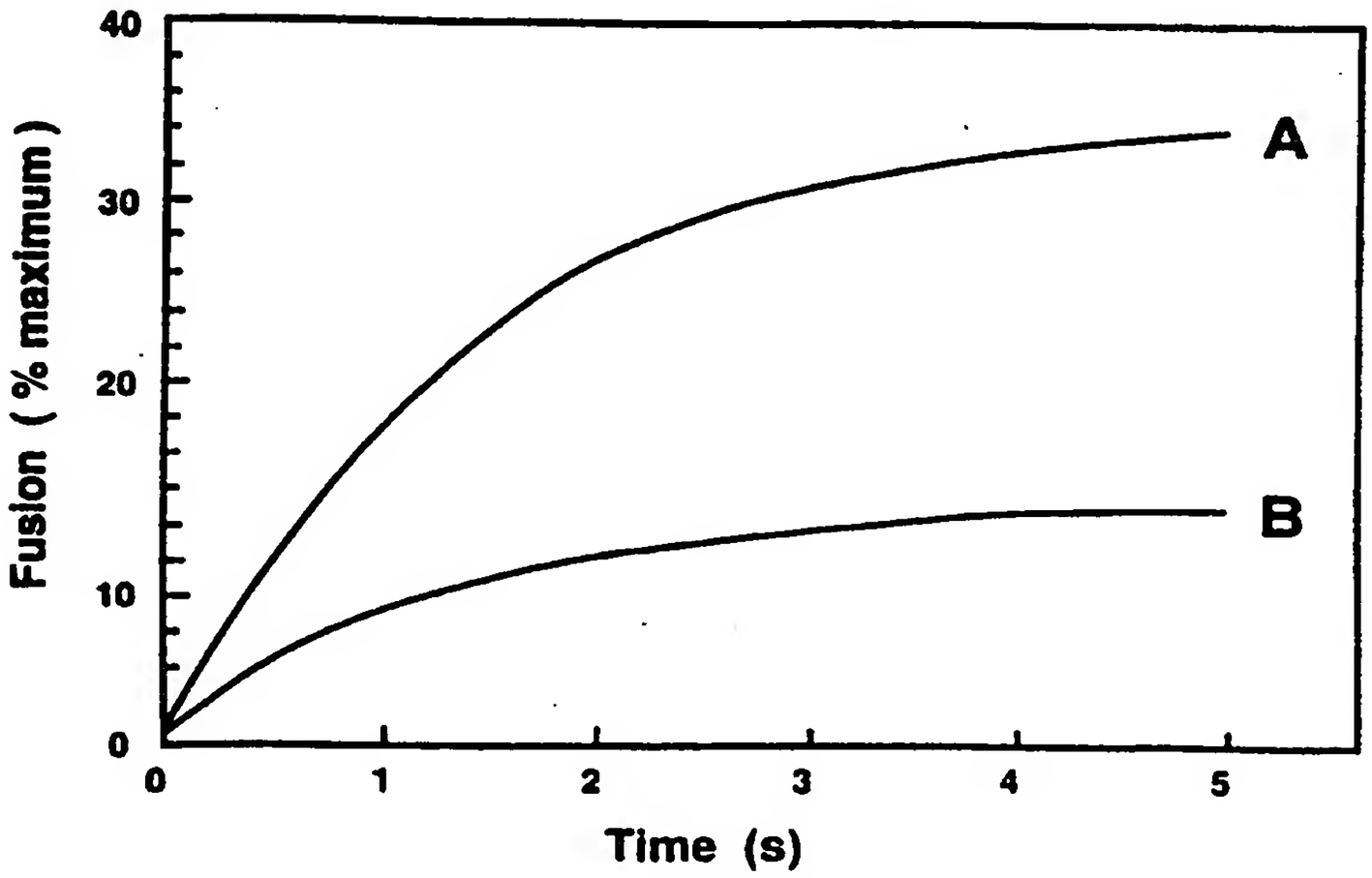
1
2
3
4
5
Time (s)
A
B
C

Fig. 3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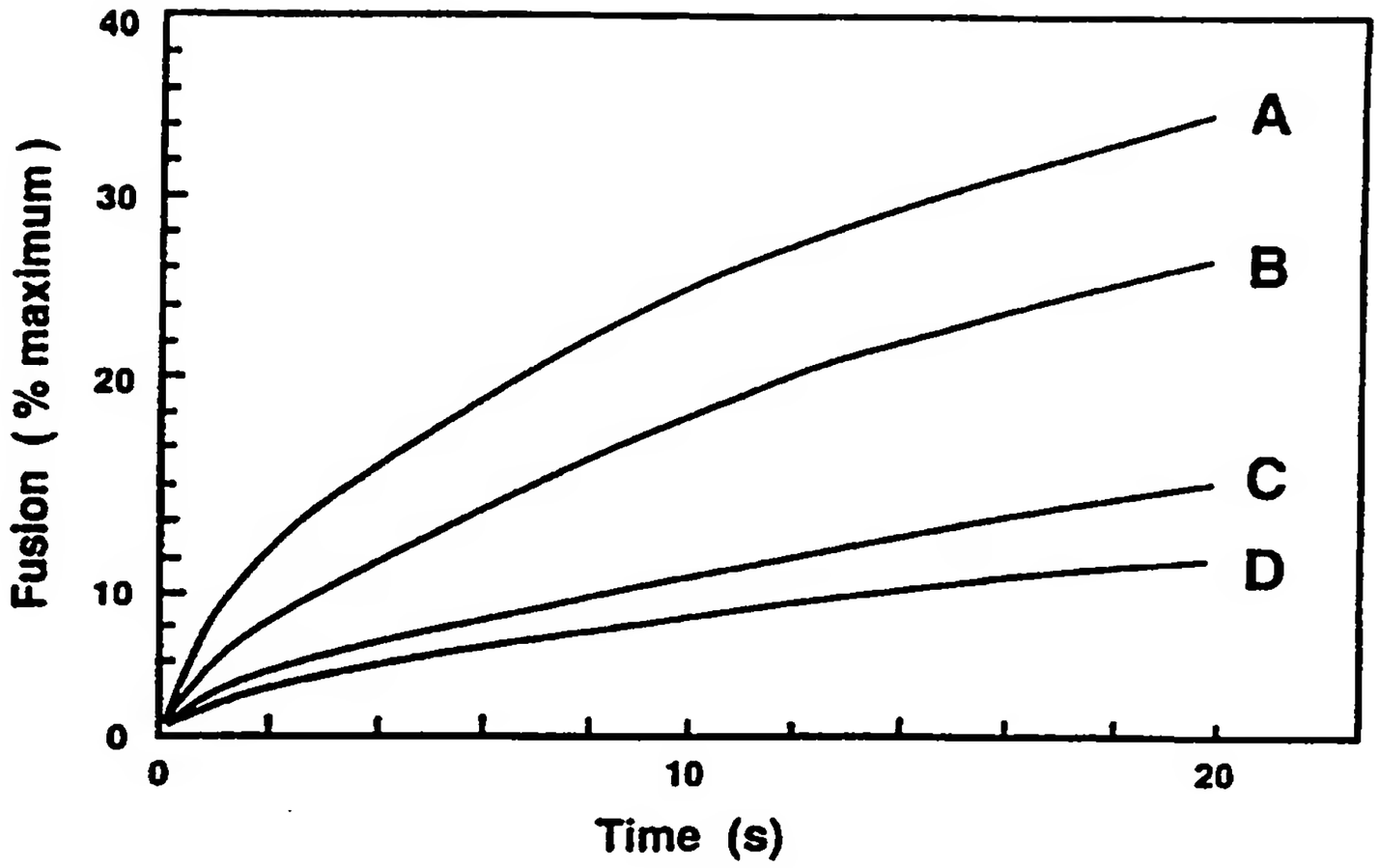
1
2
3
4
5
Time (s)
A
B

Fig. 4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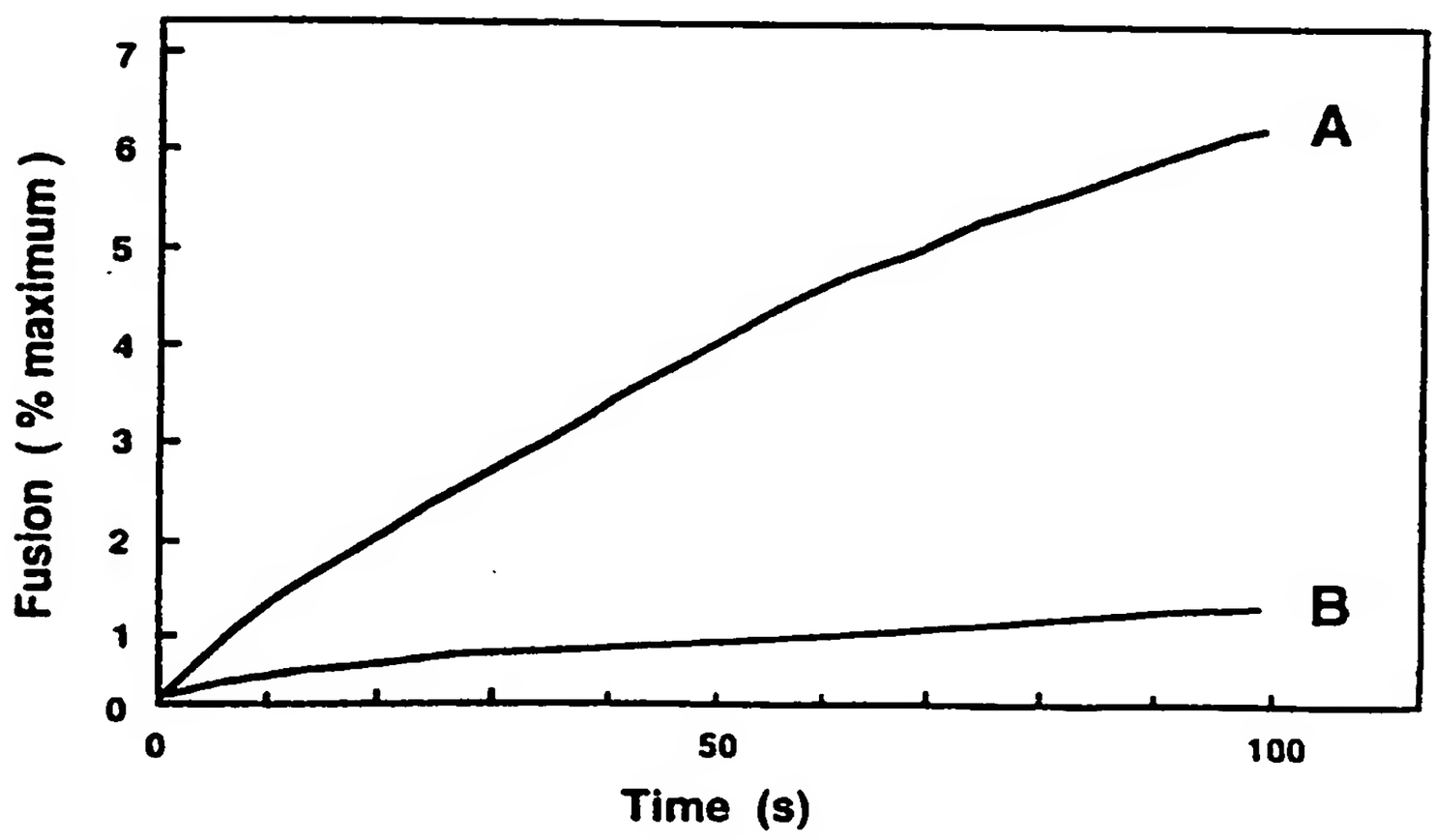
0
10
20
Fusion (% maximum)
Time (s)
A
B
C
D

Fig. 5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6
5
4
3
2
1
0
0
50
100
Fusion (% maximum)
Time (s)
A
B

Fig. 6

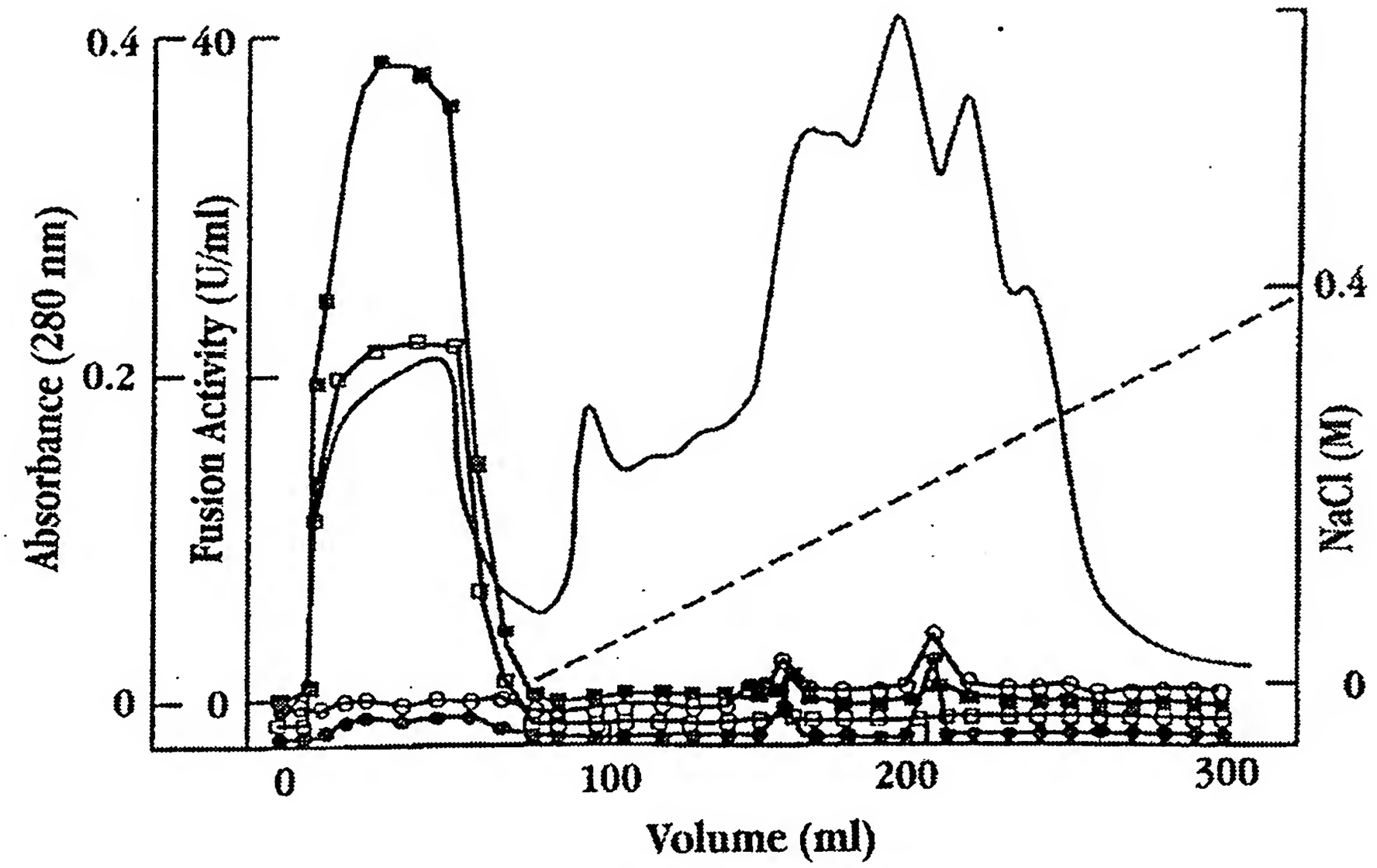


Fig. 7

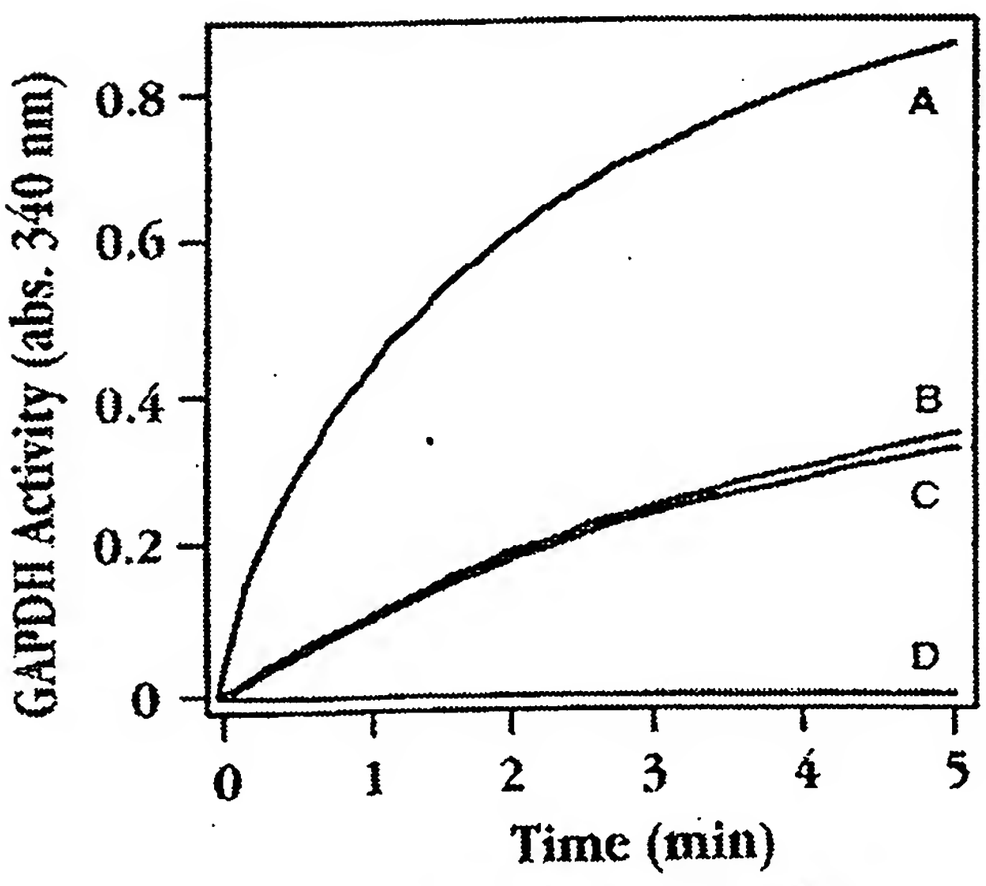


Fig. 9A

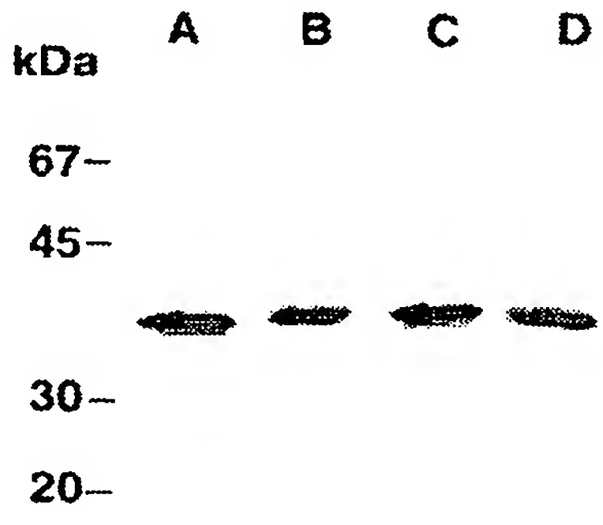
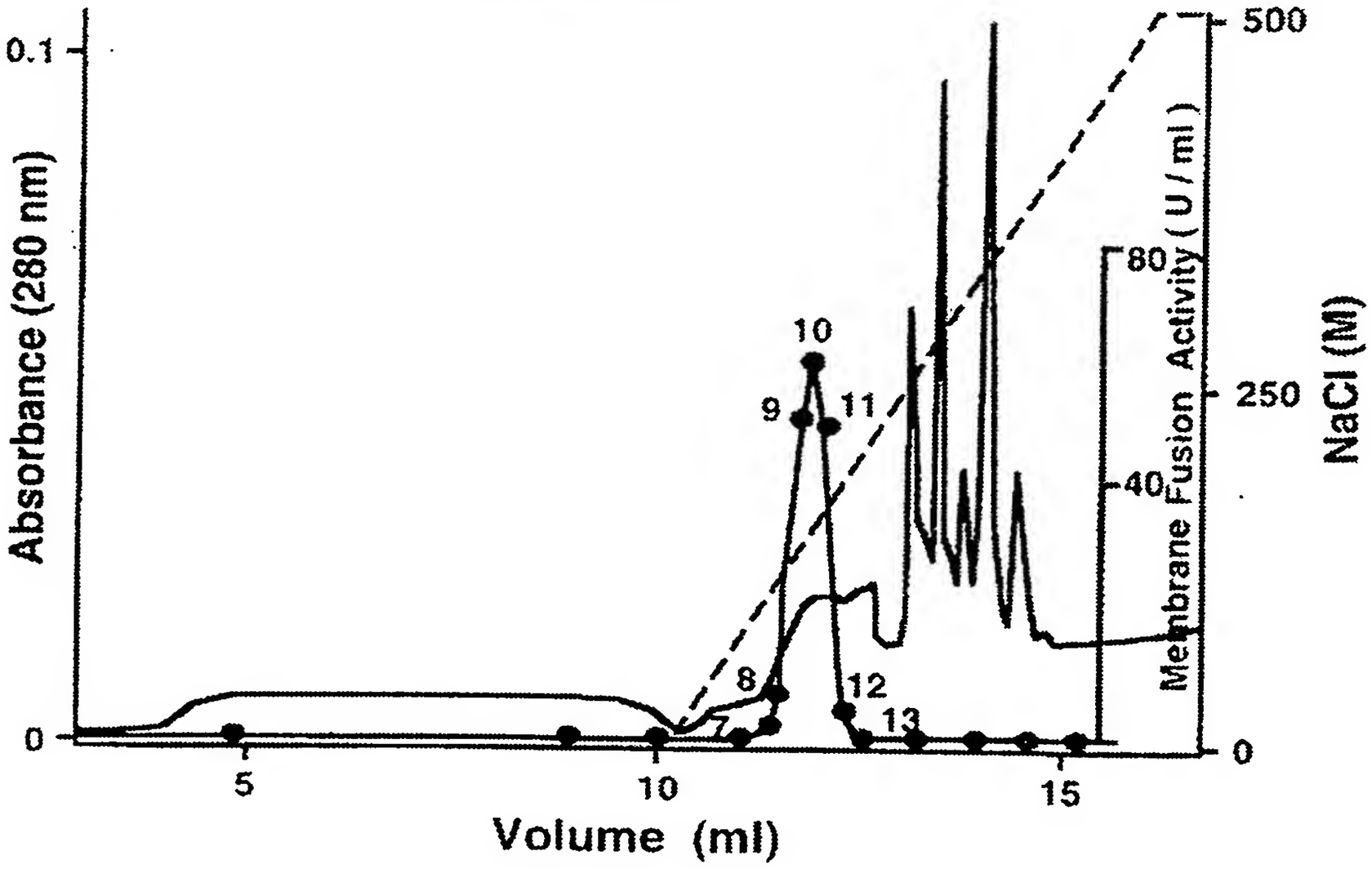


Fig. 9B

Fig. 8A



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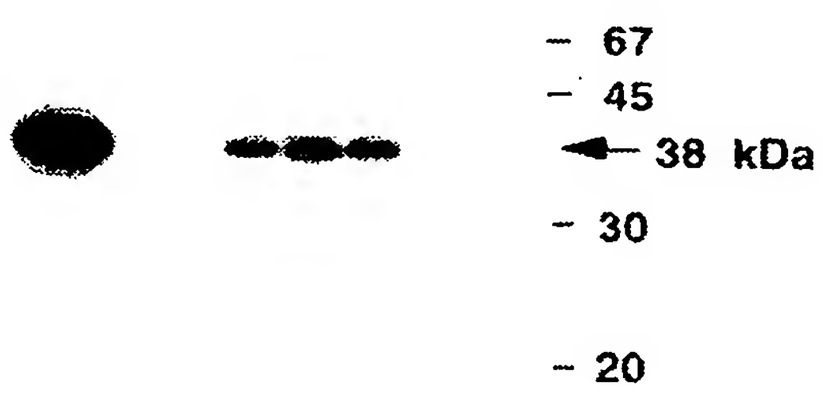


Fig. 8B

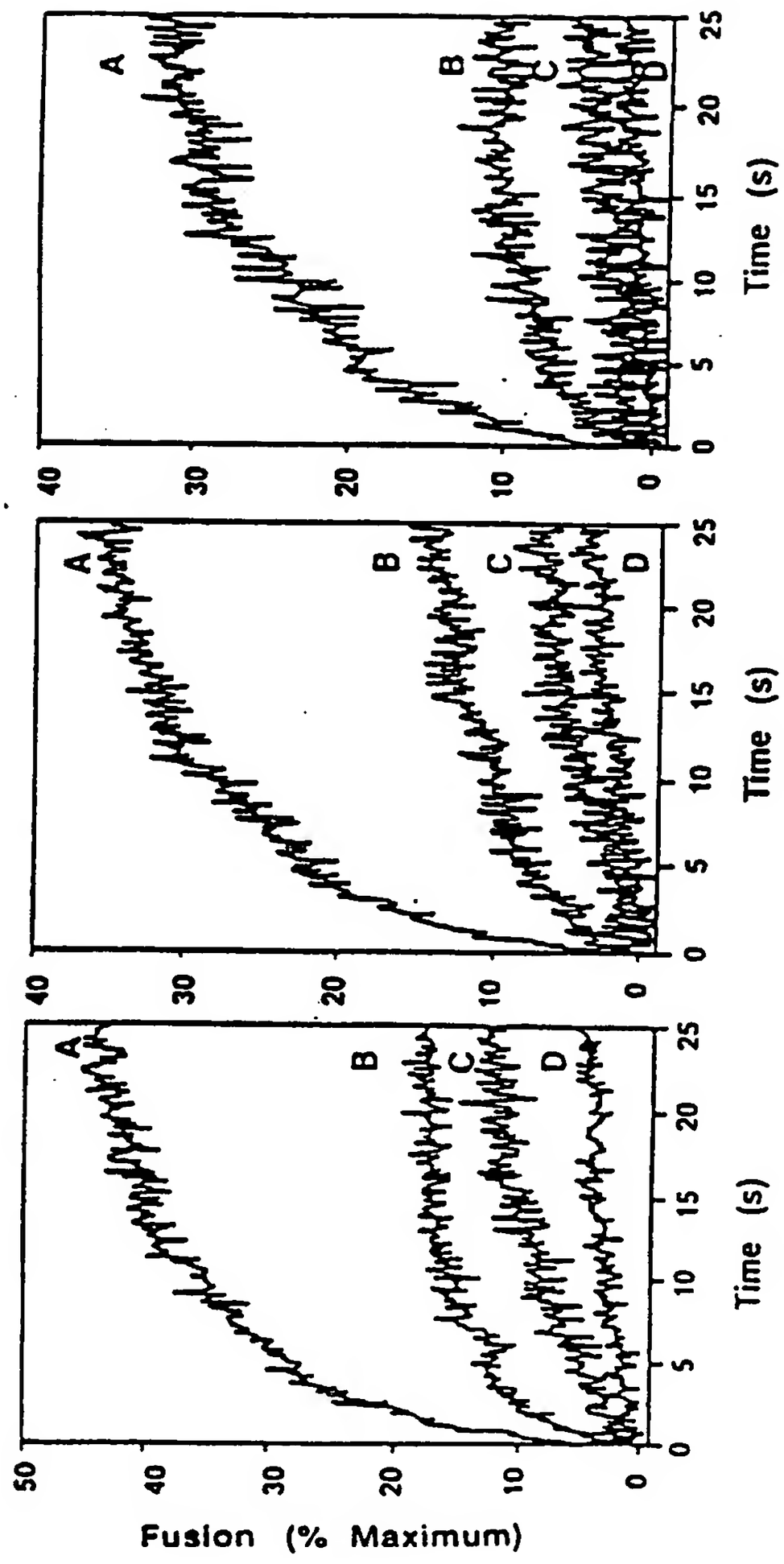


Fig. 10C

Fig. 10B

Fig. 10A

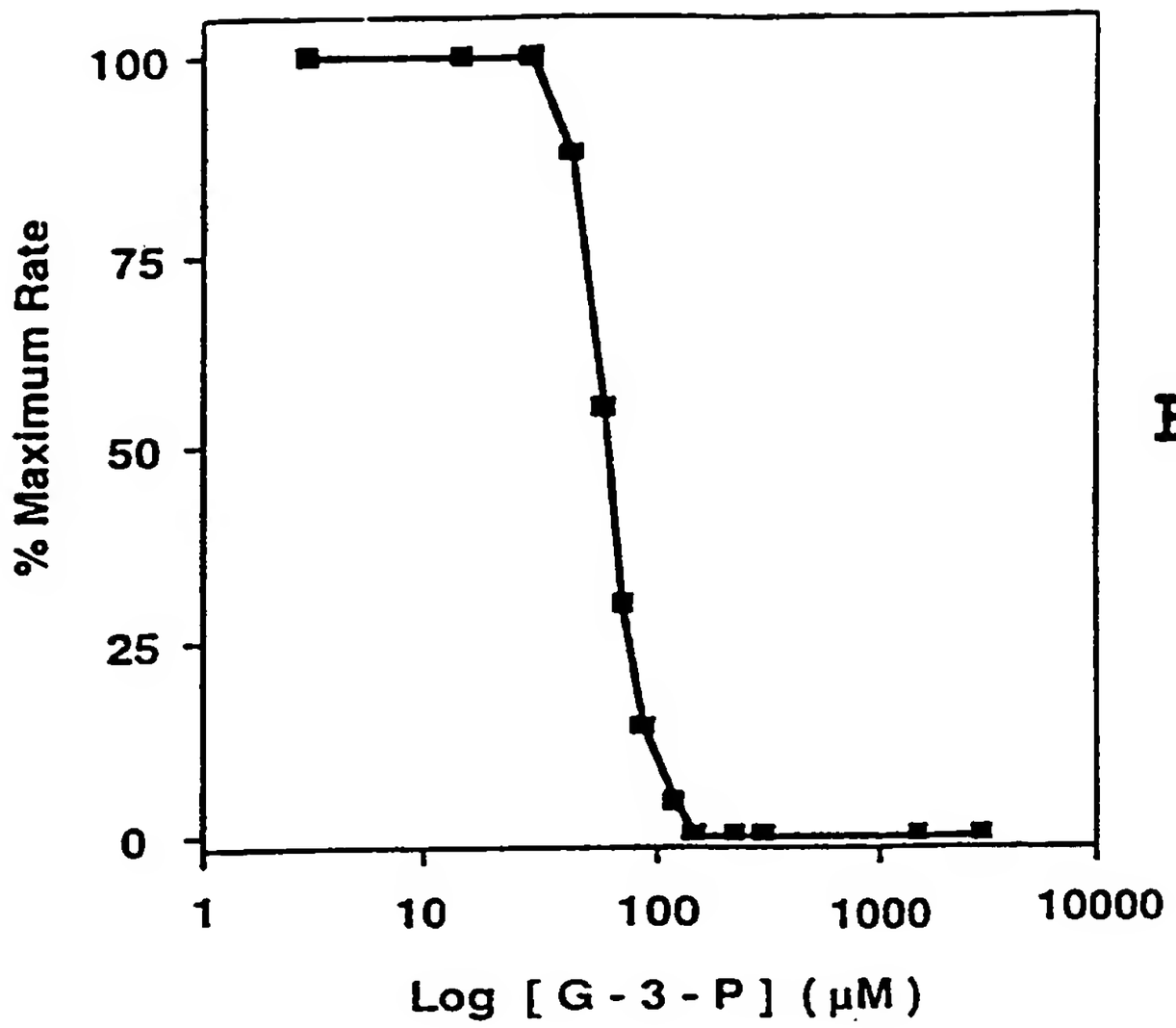


Fig. 11

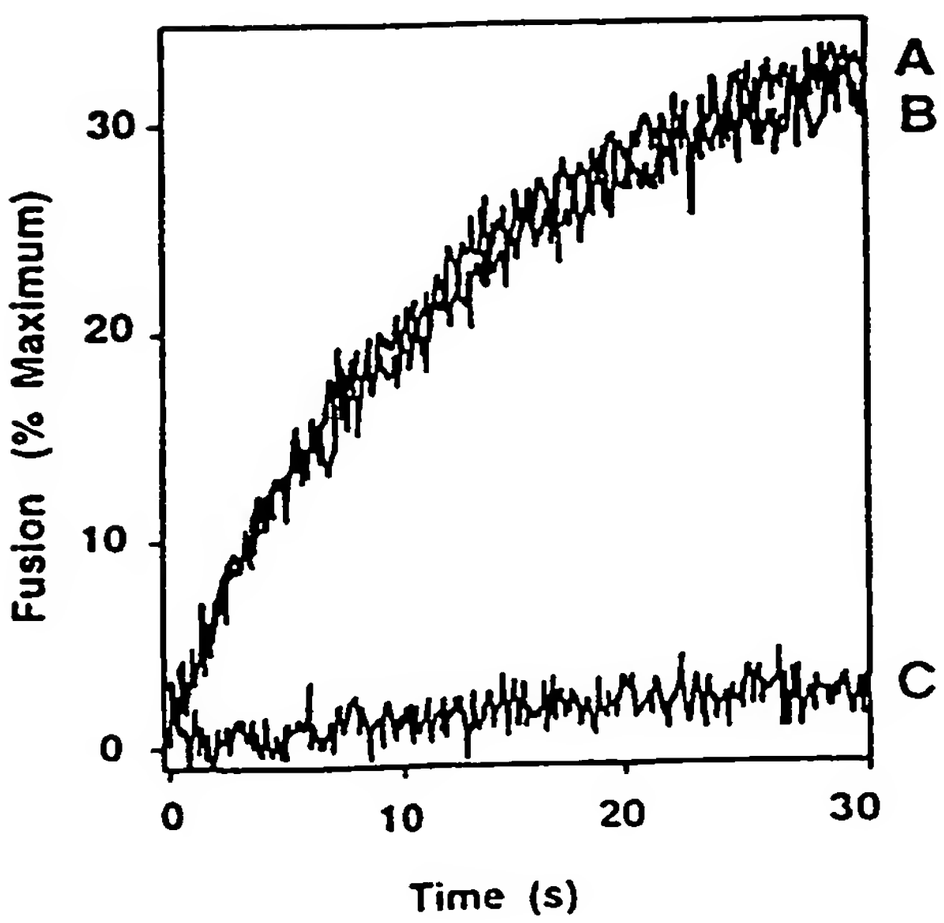


Fig. 12A

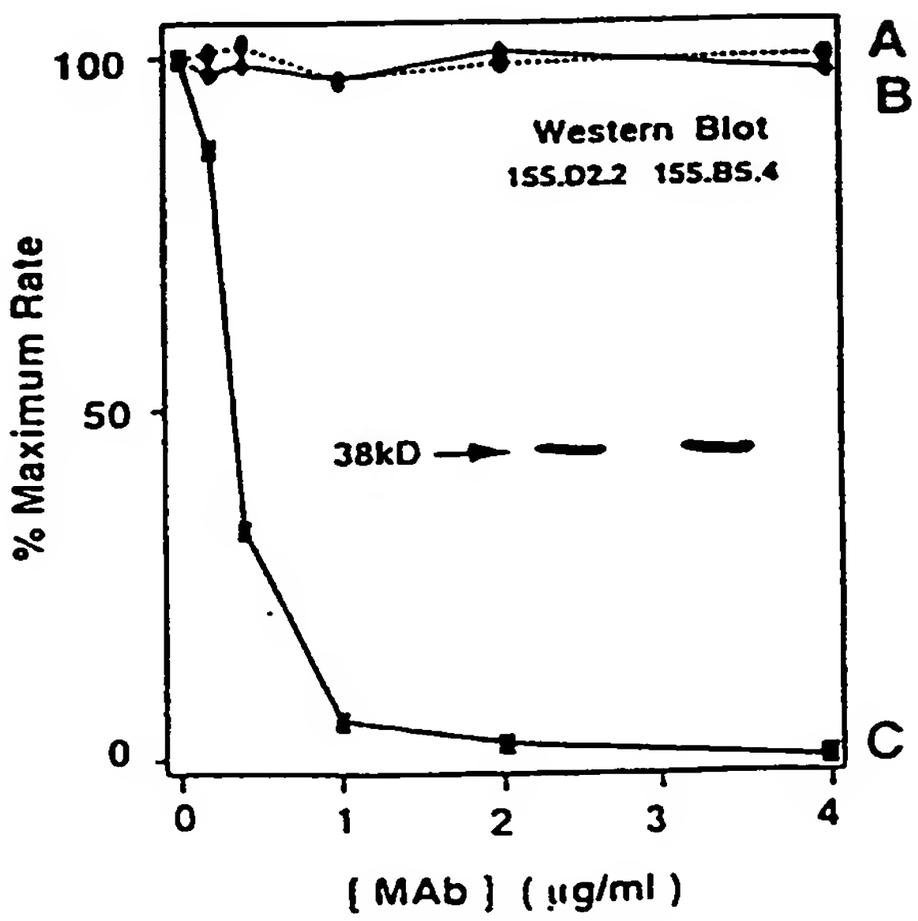


Fig. 12B

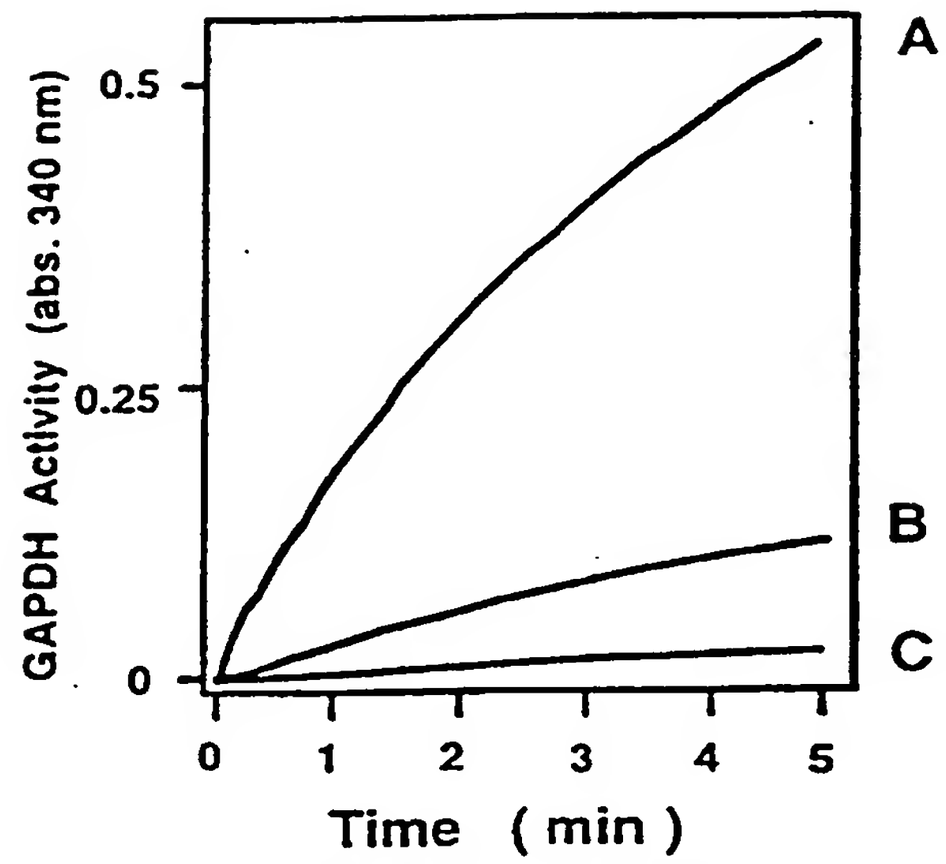


Fig. 13A

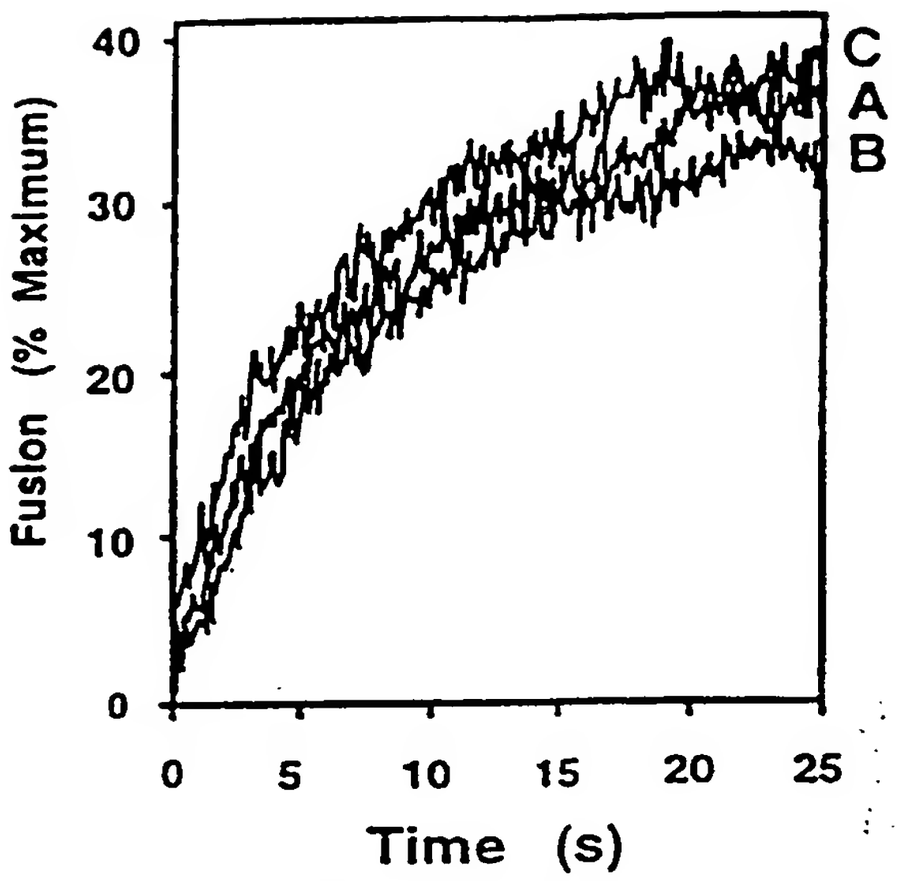


Fig. 13B

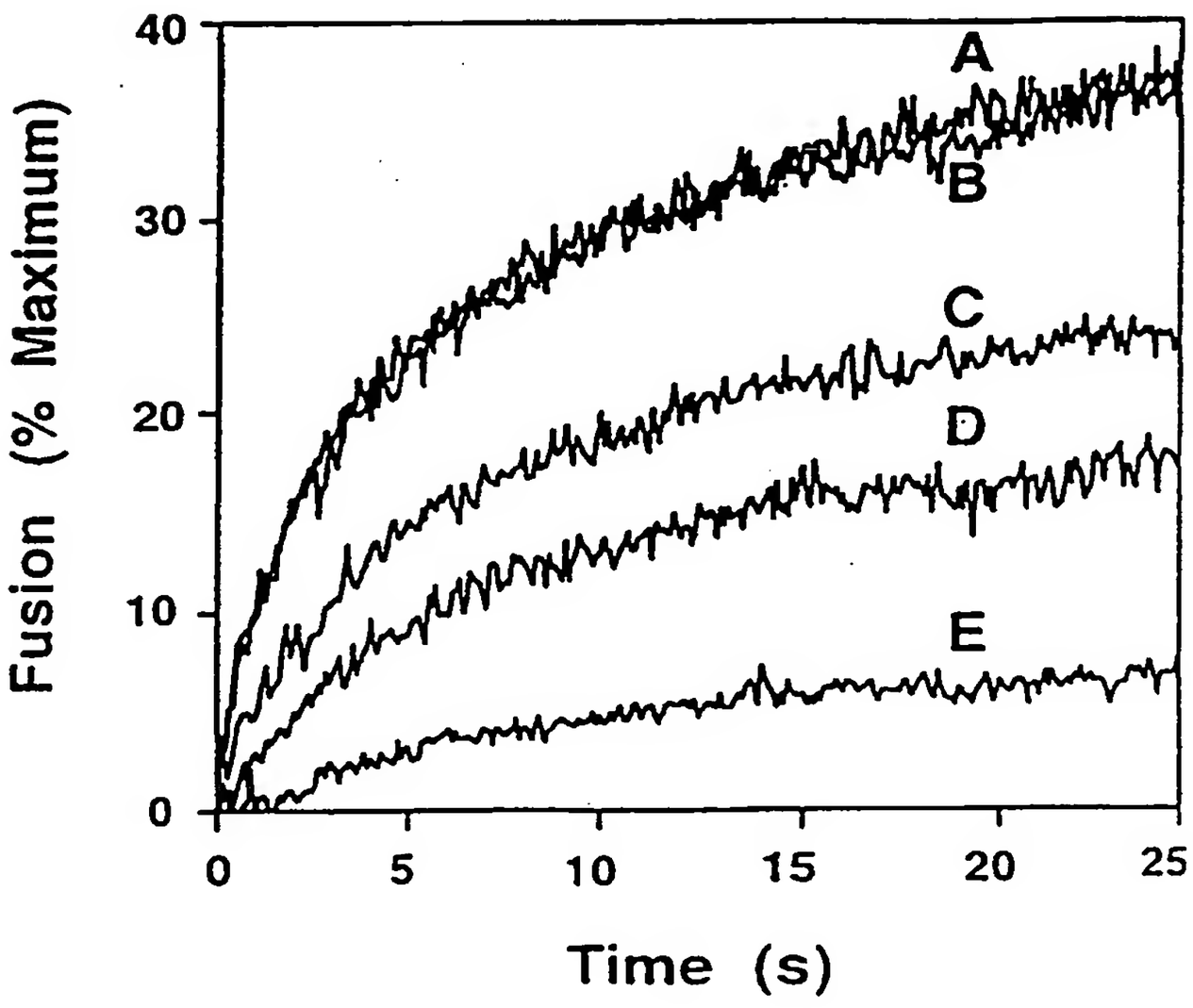
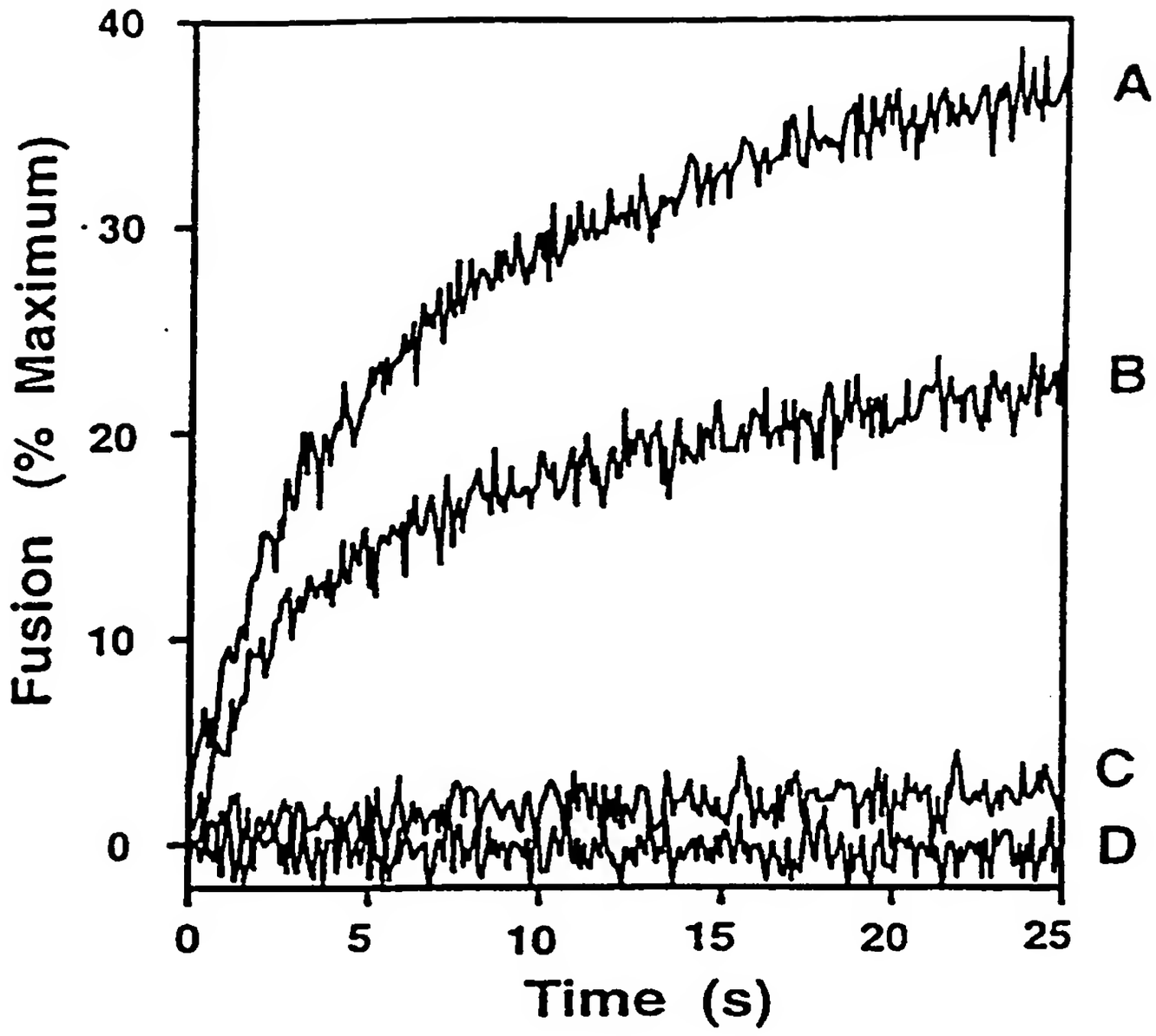


Fig. 14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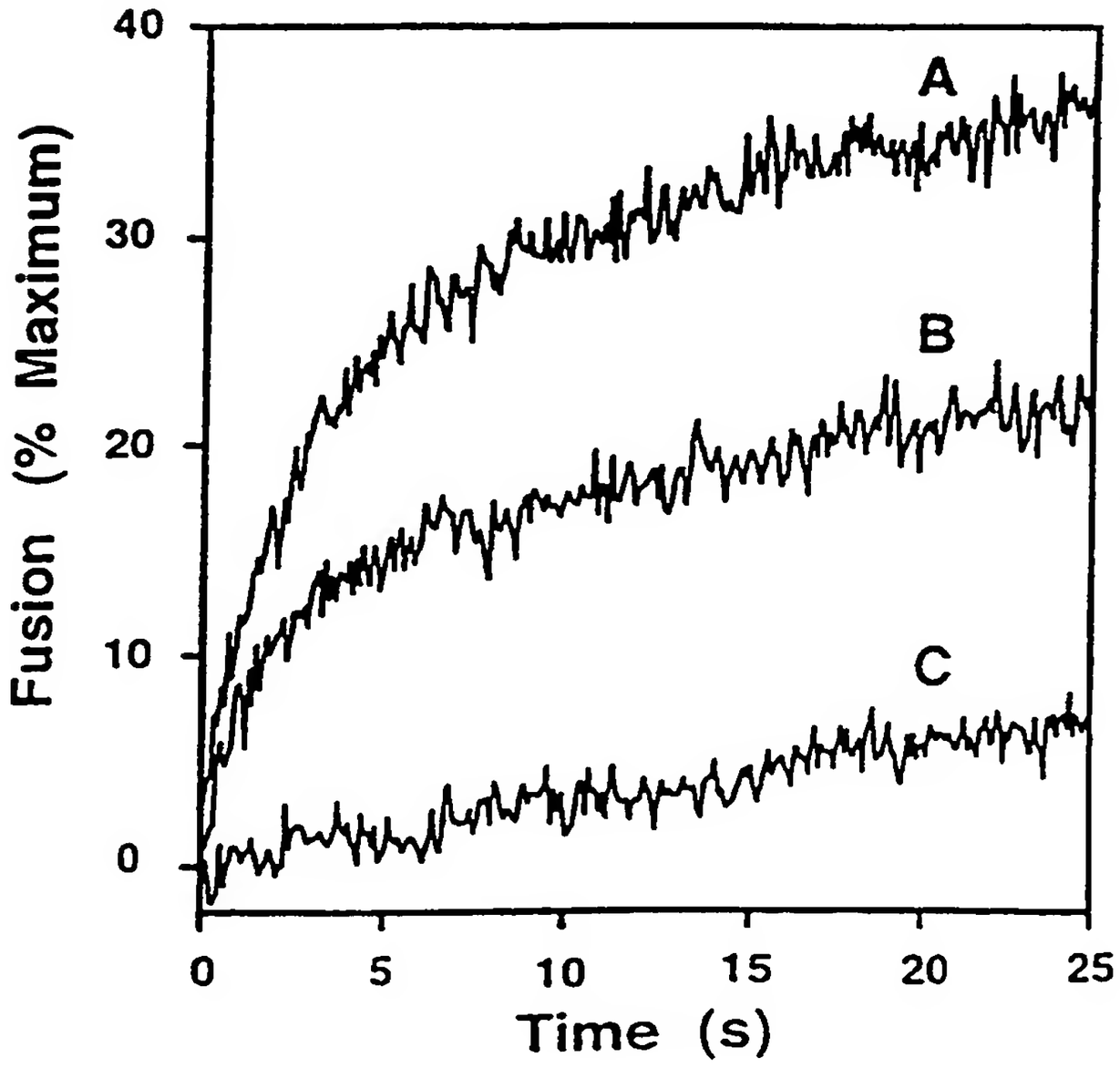
20
10
0
Fusion (% Maximum)
0
5
10
15
20
25
Time (s)
A
B
C
D

Fig. 15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30
20
10
0
Fusion (% Maximum)
0
5
10
15
20
25
Time (s)
A
B
C

Fig. 16

INTERNATIONAL SEARCH REPORT

I. ational Application No
PCT/US 95/06056

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 A61K9/127 C12N9/02

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 A61K C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO,A,93 04673 (OREGON GRADUATE INSTITUTE OF SCIENCE AND TECHNOLOGY) 18 March 1993 see claims 1,2,9-16,32 ---	15
A	BIOCHEMISTRY, vol. 24, no. 8, 9 April 1985 EASTON (US), pages 1904-1909, R.D. MORERO ET AL. 'FUSION OF PHOSPHOLIPID VESICLES INDUCED BY MUSCLE GLYCERALDEHYDE-3-PHOSPHATE DEHYDROGENASE IN THE ABSENCE OF CALCIUM' see page 1904, paragraph 1 ---	1,2,9, 18,23, 25,26

-/--

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

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"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

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"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
7 September 1995	21.09.95
Name and mailing address of the ISA European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax (+31-70) 340-3016	Authorized officer Benz, K

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INTERNATIONAL SEARCH REPORT

Ir .tional Application No
PCT/US 95/06056

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	NATURE, vol. 339, 1 June 1989 LONDON (GB), pages 355-359, D.W. WILSON ET AL. 'A FUSION PROTEIN REQUIRED FOR VESICLE-MEDIATED TRANSPORT IN BOTH MAMMALIAN CELLS AND YEAST' cited in the application see page 355, column 1, paragraph 1 ---	10,24
A	US,A,4 873 089 (SCOTTO ET AL.) 10 October 1989 see the whole document -----	1

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INTERNATIONAL SEARCH REPORT

Information on patent family members

In tional Application No
PCT/US 95/06056

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
WO-A-9304673	18-03-93	US-A-	5277913	11-01-94
		AU-A-	2590292	05-04-93
		JP-T-	7502261	09-03-95
US-A-4873089	10-10-89	NONE		